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Efficient production of anti-fluorescein and anti-lysozyme as single-chain anti-body fragments (scFv) by *Brevibacillus* expression system

Hiromasa Onishi [a], Makoto Mizukami [a], Hiroshi Hanagata [a], Masao Tokunaga [b], Tsutomu Arakawa [c], Akira Miyauchi [a,*]

[a] Bio Research Group, R&D Department, Higeta Shoyu Co., Ltd., 2-8 Chuo-cho, Choshi, Chiba 288-8680, Japan
[b] Applied and Molecular Microbiology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan
[c] Alliance Protein Laboratories, 6042 Cornerstone Court West, Suite A, San Diego, CA 92121, USA

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ABSTRACT

Expression of scFv in *Brevibacillus choshinensis* was tested using combinations of three different promoters and four different secretion signals. Two model scFv constructs, i.e., His-scFvFLU and His-scFvHEL, were successfully expressed with some of the combinations. Ni Sepharose column and size exclusion chromatography resulted in fairly pure preparations of these two proteins. The purified His-scFvFLU inhibited fluorescence from fluorescein, while the purified His-scFvHEL inhibited lysozyme activity. Relatively high yield of His-scFvFLU (~40%) and His-scFvHEL (~30%) was achieved with the expression and purification system described here.

Introduction

Antibody fragments, such as scFv, Fab and diabody, are under intense development in various therapeutic applications, in particular in the area of immunotoxins [1–10]. Unlike intact antibodies, however, antibody fragments are not readily amenable to a platform standard mammalian expression system [11–13]. Refolding of insoluble protein from *Escherichia coli* is also often used, requiring extensive trial-and-error approaches [14–19]. Even when mammalian expression system works, yield is often low with high cost. Here we have used *Brevibacillus choshinensis* secretory expression system for 2 model scFv proteins.

B. choshinensis is related to *Bacillus*, a single cell wall gram-positive bacterium. Both *B. choshinensis* and *Bacillus brevis* produce a large quantity of cell wall proteins that are shed into the culture media. Such high expression of cell wall proteins is due to strong and novel promoters [20]. Exploiting this unique feature, we constructed a host-vector system using *B. choshinensis* as a host, especially suited for secretory production of recombinant proteins. The host bacterium, *B. choshinensis* HPD31-SP3, has been genetically modified to prevent spore formation that can potentially cause contamination problem. In addition, weak protease activity of this host cell has been reduced to a practically negligible level by disrupting 2 major protease genes. With these genetic manipulations, this expression system has a long record of successful production of recombinant proteins [21]. Recently, we developed an easy plasmid construction method, "*Brevibacillus in vivo* cloning (BIC)[1]". Thus, *in vivo* homologous recombination of linearized vector and insert DNA fragments with overlapping ~15 bp sequences at both 5′-and 3′-ends resulted in construction of proper expression vector in the *Brevibacillus* competent cells. Once several linearized vector cassettes were prepared, target gene were inserted by PCR amplification with overlapping short sequences at both ends and simply by transformation of both vector and insert fragments at once to competent cells [22]. In the present study, we examined combinations of 3 promoters and 4 signal sequences for secretory expression of scFv with amino-terminal His-tag (His-scFv) and succeeded in producing two active His-scFv proteins.

* Corresponding author. Fax: +81 479 24 3422.
E-mail address: a-miyauchi@higeta.co.jp (A. Miyauchi).

[1] *Abbreviations used:* BIC, *Brevibacillus in vivo* cloning; scFvHEL, HyHEL-10 single chain scFv; SEC, size exclusion chromatography.

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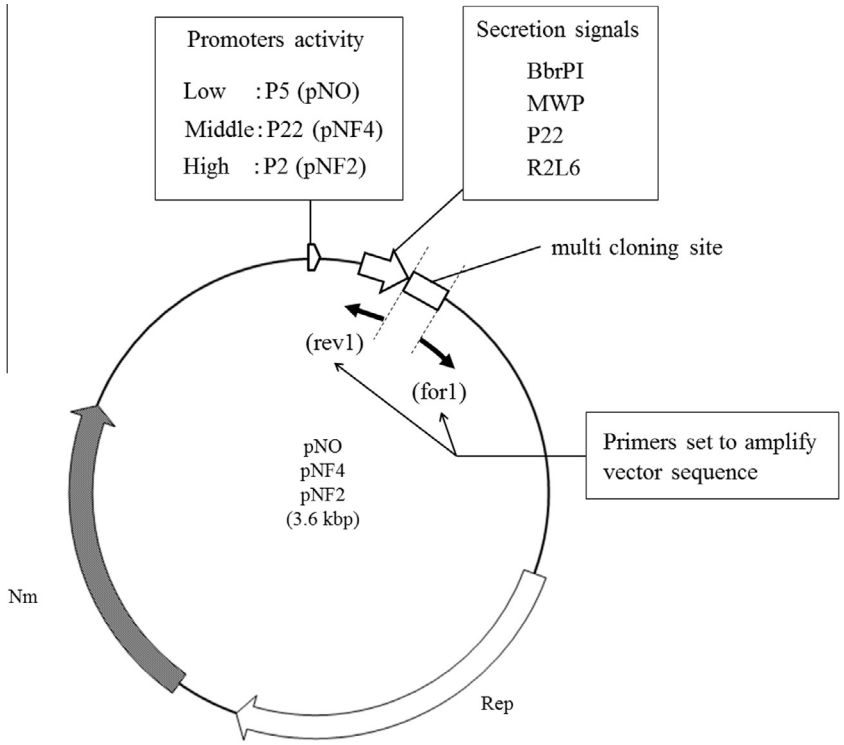

Fig. 1. Structure of 12 expression vectors with combination of 3 promoters and 4 secretion signals. Three kinds of promoters and 4 secretion signals are shown. Primer positions (for and rev) to amplify vector sequences are also shown by arrows. Nm, neomycin resistant gene; Rep, gene for plasmid replication.

Table 1
Primer sets for amplification of signal sequences.

Secretion signals	Primer sequences
BbrPI	Forward (for2): 5′-gaacacaaggtcatgaaaacaatacgaacagg-3′
	Reverse (rev2): 5′-catcctgttaagctttgccgataccacgttggttc-3′
MWP	Forward (for3): 5′-gaacacaaggtcatgaaaaaggtcgttaacag-3′
	Reverse (rev3): 5′-catcctgttaagctttgcgaaagccattggagcaa-3′
P22	Forward (for4): 5′-gaacacaaggtcatgtcaatttcggtaagatt-3′
	Reverse (rev4): 5′-catcctgttaagctttgcgaatgcggaacttggta-3′

Those 15 bp nucleotides underlined overlap with those of each of the vector's 5′ and 3′ ends.

Table 2
Primer sets for 4 backbone secretion vectors.

Secretion signals	Primer sequences
BbrPI signal vector	Forward (for1): 5′-aagcttaacaggatg-3′
	Reverse (rev5): 5′-tgccgataccacgttggttc-3′
MWP signal vector	Forward (for1): 5′-aagcttaacaggatg-3′
	Reverse (rev6): 5′-tgcgaaagccattggagcaa-3′
P22 signal vector	Forward (for1): 5′-aagcttaacaggatg-3′
	Reverse (rev7): 5′-tgcgaatgcggaacttggta-3′
R2L6 signal vector*	Forward (for1): 5′-aagcttaacaggatg-3′
	Reverse (rev8): 5′-agcgaaagccatgggagcaa-3′

* R2L6 signal is the original secretion signal for pNO, pNF4, pNF2 vectors.

Materials and methods

Bacterial strain and culture medium

B. choshinensis strain HPD31-SP3 (FERM BP-8479) was used as a host bacterium. *B. choshinensis* was grown in 2SL medium. 2SL medium contained 2% glucose, 4% Polypeptone S (Nihon Pharmaceuticals, Tokyo, Japan), 0.5% yeast extract BSP-B (Oriental yeast Co., Ltd., Tokyo, Japan), 0.001% $FeSO_4{\cdot}7H_2O$, 0.001% $MnSO_4{\cdot}4H_2O$, 0.0001% $ZnSO_4{\cdot}7H_2O$, pH 7.2, and 50 μg/ml neomycin. Glucose was sterilized separately.

Construction of Brevibacillus expression plasmids

We have examined two scFv genes, HyHEL-10 single chain scFv (scFvHEL) derived from anti-lysozyme monoclonal antibody [23] and 4M5.3 single chain scFv (scFvFLU) developed against fluorescein [24].

Three plasmids (i.e., pNO, pNF4 and pNF2) containing a promoter with low (P5), middle (P22) or high (P2) promoter activity [20] and a signal sequence R2L6 [25] were used to construct expression vectors for His-scFv proteins. R2L6 secretion signal was replaced by three different signal sequences, BbrPI [26], MWP [20] and P22, to prepare expression vectors with 4 secretion signals in total (i.e., BbrPI, MWP, P22 and original R2L6, Fig. 1). The primer sets shown in Table 1 were used to amplify 3 nucleotide sequences, i.e., BbrPI, MWP and P22 signals. BbrPI and MWP have been reported elsewhere [20,26]. P22 is a 22 kDa protein efficiently secreted by *B. choshinensis*. Similarly, vector sequences without signal sequences were amplified from pNO, pNF4 and pNF2 vectors using primer sets, for1 (5′-aagcttaacaggatg-3′) and rev1 (5′-catgaccttgtgttctcctc-3′). DNA fragments encoding signal sequences and vector sequences thus amplified were transformed at once into *B. choshinensis* competent cells. The vectors and the signal sequences simultaneously transformed were designed to