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REPORT

Comprehensive study of domain rearrangements of single-chain bispecific antibodies to determine the best combination of configurations and microbial host cells

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ABSTRACT

Small bispecific antibodies (bsAbs) are important therapeutic molecules and represent the first bsAb format approved by the United States Food and Drug Administration. Diabody (Db), a small bsAb format, has four possible domain orders; we previously reported the differences in the expression levels and cancer growth inhibition effects upon rearranging the domain order of this format. However, there have been no comprehensive reports on domain rearrangements of bispecific single-chain Db (scDb) and tandem single-chain Fv (taFv), which are widely used bsAb formats. In this study, we designed all possible domain orders for scDb and taFv (each with eight variants) with identical Fv pairs and individually expressed all 16 variants using *Escherichia coli*, *Pichia pastoris*, and *Brevibacillus choshinensis*. Comprehensive investigations showed that the intrinsic functions of the variants were similar to each other, regardless of the expression host system, but expression levels varied depending on the format as well as on the host cell. Among the 16 variants, we found a promising candidate that exhibited high activity and productivity. Furthermore, we determined that *B. choshinensis* is an attractive expression host because of its secretory production of recombinant proteins.

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Introduction

Conventional monoclonal therapeutic antibodies have been widely used to treat a variety of diseases that are difficult to cure[1,2]; however, production costs for those produced in mammalian expression systems can be high. In addition, especially in cancer treatments, adverse clinical outcomes and data from animal studies have highlighted important limitations in their modes of action and therapeutic efficacy.[3] Therefore, many strategies to improve the functions of antibodies have been explored; one such strategy is the design of non-natural antibody formats, particularly bispecific antibodies (bsAbs). These antibodies are characterized by their ability to simultaneously bind two targets, which, for example, allows effective redirection of diverse effectors such as cytotoxic T cells toward cancer cells. Blinatumomab (Blincyto®) was the first, and so far only, bispecific T-cell redirecting therapeutic antibody drug approved by the United States Food and Drug Administration.[3]

Advances in recombinant technology have made it feasible to generate small recombinant bsAb constructed from two different variable antibody fragments, such as variable fragments (Fvs), single-chain Fvs (scFvs), and variable domains of the heavy chain of heavy-chain antibodies (VHH). These recombinant bsAbs include diabodies (Dbs),[4] single-chain diabodies (scDbs),[5] tandem scFvs (taFvs),[6] minibodies (dimeric scDb-CH3 fusion proteins),[7] and tandem VHH.[8] In contrast with classic bsAbs prepared through chemical conjugation or quadroma production,[9–11] small bsAbs may allow greater tissue penetration and high target retention, and they are can be produced using cost-efficient microbial expression systems.[12,13] Hosts such as *Escherichia coli*[14] and *Pichia pastoris*[15] have been used to produce small bsAbs; however, clinical-grade production using these hosts is still challenging. In fact, blinatumomab was produced in Chinese hamster ovary cells, i.e., a mammalian expression system.

Brevibacillus choshinensis was originally isolated from soil and found to secrete large quantities of protein with low extracellular protease activity.[16] This nonpathogenic Gram-positive bacterium can secrete recombinant proteins in culture directly without concern for contaminating endotoxins, facilitating the purification process.[17,18] *B. choshinensis* has been used as a host for the production of industrial proteins as well as for biological products. Production of human epidermal growth factor (EGF) was reported in reclassified strain *B. choshinensis* HPD31;[19] however, there have been no reports of the production of bsAbs using this microorganism.

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Several reports have suggested that the order of the variable domain affected the expression level and the function of small bsAb fragments such as bispecific taFv and Db.[20,21] We previously described the construction of a functional humanized bispecific Db (bsDb) that targeted human EGF receptor (EGFR) and CD3 (hEx3-Db).[22] The bsDb format has four possible domain orders, and we reported the differences in the expression levels and cancer growth inhibition effects by rearranging the domain order.[23] Although the bsDb format has a potential concern regarding the formation of inactive homodimers concurrent with the active heterodimers, construction of scDb by connecting an additional middle linker can solve this problem. However, bispecific scDb and taFv formats have eight possible domain orders, and the effects of domain order on the expression levels and the function of these formats have not been investigated yet.

In this study, we constructed expression vectors for all possible domain orders of scDb and taFv formats of hEx3. We prepared 16 hEx3 variants using *E. coli, P. pastoris*, and *B. choshinensis* expression systems. Finally, we evaluated and compared their expression levels and function. To our knowledge, this is the first report on the preparation and evaluation of all configurations of bispecific scDb and taFv comprising identical Fv pairs, as well as of the production of bsAb in *B. choshinensis*. Our results showed that the intrinsic functions of hEx3 in each format were similar regardless of host cells used, but the expression levels varied in each format in each host cell. Furthermore, we found that *B. choshinensis* is a good candidate expression host for the secretory production of small bsAb.

Results

Design and construction of expression vectors for hEx3-scDbs and -taFvs with different domain orders

To comprehensively investigate the effects of rearranging the domain order on the functions of small bsAbs, we designed all possible domain orders for the single-chain bsAb format of hEx3, resulting in eight types of hEx3-scDb and eight types of hEx3-taFv. We also constructed expression vectors for these variants. Schematic diagrams of the gene constructs of all 16 types of hEx3 bsAbs are summarized in Figure 1. Because of the similarity in the codon used by *E. coli* and *B. choshinensis*, whole genes optimized for *E. coli* codons were synthesized and used for the construction of the expression vectors for both *E. coli* and *B. choshinensis*. In contrast, genes optimized for *P. pastoris* codons were used for the construction of vectors for the yeast expression system. We effectively constructed 48 whole-gene expression vectors accounting for 16 hEx3 variants for each of the three host cells.

Evaluation of bsAbs expressed in E. coli

To evaluate the influence of the domain order of hEx3 bsAb on the inhibition of human carcinoma cell growth, we prepared 16 hEx3 variants using *E. coli* as the expression system and conducted analysis using 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Western blot analysis of the culture supernatant showed that there were differences in the expression levels in each variant (Figure 2a,b). We also directly used each culture supernatant to evaluate cancer growth inhibition effects.[24] In brief, we used culture supernatant diluted more than 10-fold, which showed no background cytotoxicity. Optimally, biologicals should be both highly effective and easily produced in large quantities. The scDb formats sc1, sc3, sc5, and sc8 were promising candidates as they showed high activity despite low expression levels. The taFv formats ta1, ta2, ta6, and ta8 were also promising candidates, with ta1 and ta2 showing high activity but low expression levels, while ta6 and ta8 showed both high activity and expression levels (Figure 2b,c).

Evaluation of bsAbs expressed in P. pastoris

To confirm the validity of our variant selection in *E. coli*, we purified bsAbs expressed in *P. pastoris*, which was expected to produce high-purity proteins in the supernatant. The expression levels estimated by western blot analysis of the culture supernatant

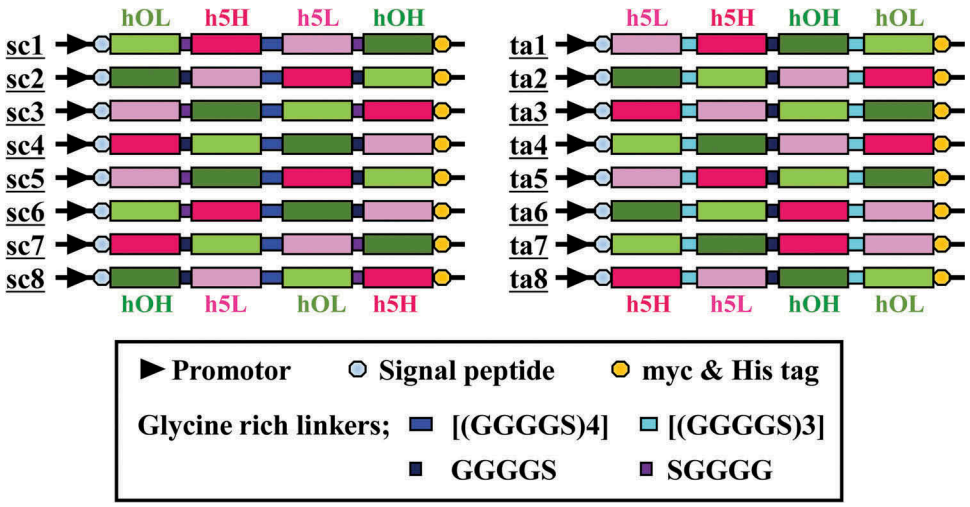

Figure 1. Schematic diagram of gene constructs of 16 hEx3 bispecific antibodies (bsAbs). The labels sc1–sc8 and ta1–ta8 correspond to single-chain Db (scDb) and tandem single-chain Fv (taFv) formats with different domain orders, respectively.

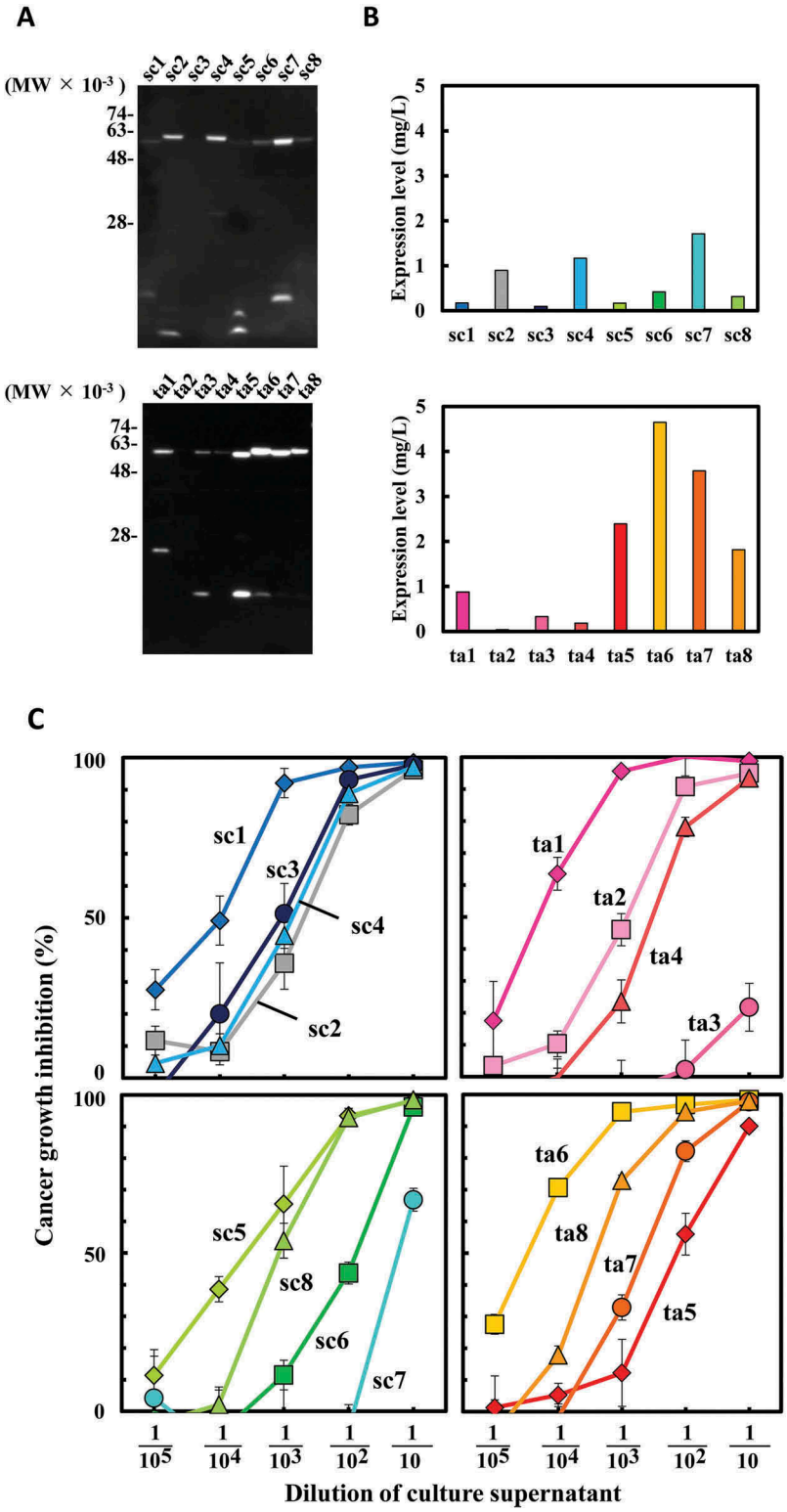

Figure 2. Expression of 16 hEx3 variants in *Escherichia coli* and their growth inhibition effects against epidermal growth factor receptor (EGFR)-positive TFK-1 cells. Expression levels were estimated by western blotting analysis of culture supernatant (a, b). Diluted culture supernatants of *E. coli* transformants and T-LAK cells were added to TFK-1 cells (T-LAK:TFK-1 ratio, 5:1) (c).

revealed differences in the pattern of expression levels in *P. pastoris* compared to *E. coli*. In *P. pastoris*, sc1 and ta6 showed moderate expression levels (Figure 3a). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis verified the single bands of each purified His-tagged bsAbs prepared from culture supernatants (Figure 3b). Growth inhibition assays using the samples showed high activities for sc1, sc3, sc5, ta1, ta2, ta6, and ta8, all promising candidates from *E. coli* expression (Figure 3c). These results indicate the applicability of using unpurified culture supernatant for rapid and easy prescreening.

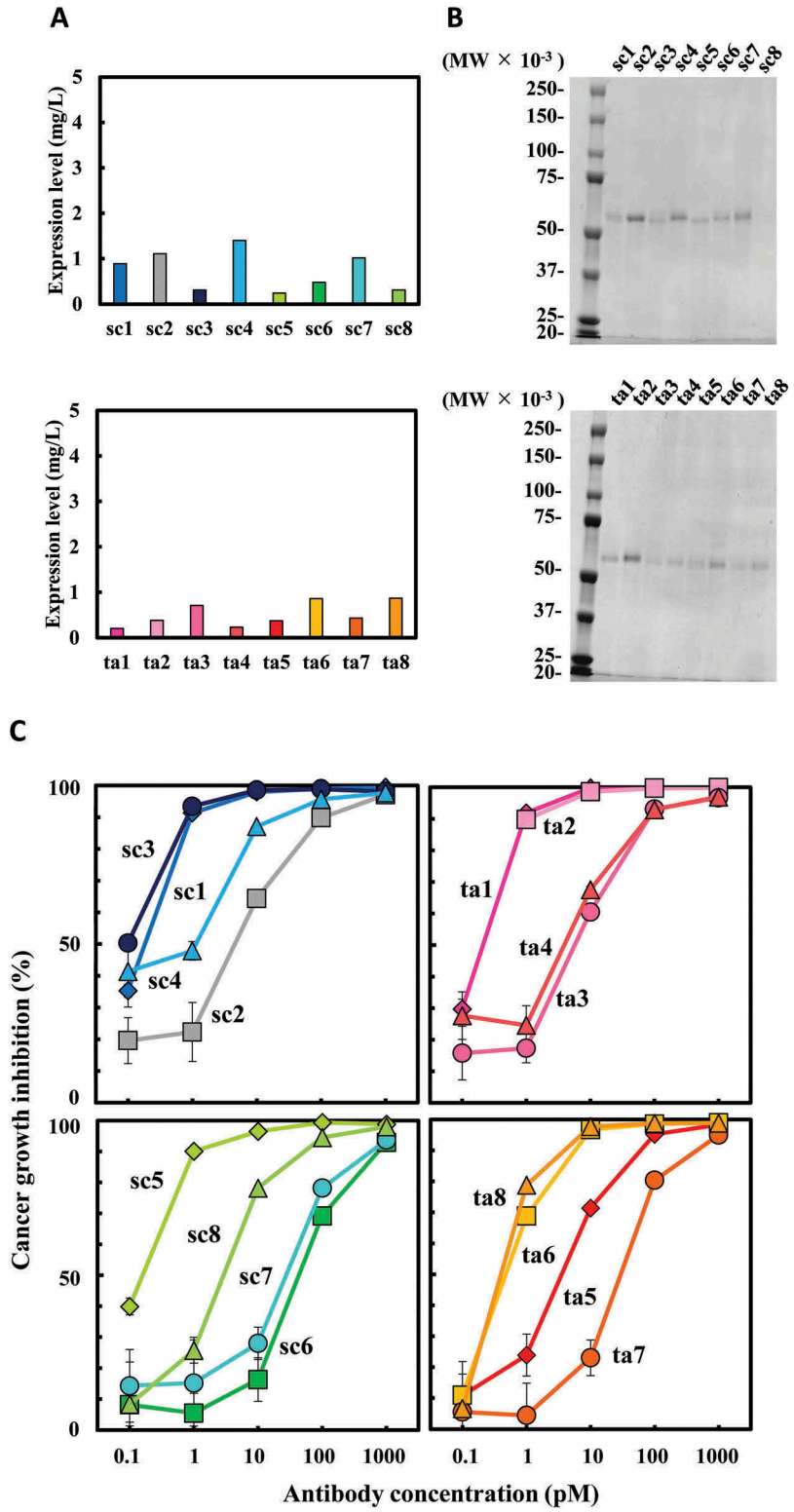

Figure 3. Expression of 16 hEx3 variants in *Pichia pastoris* and their growth inhibition effects against epidermal growth factor receptor (EGFR)-positive TFK-1 cells. Expression levels were estimated by western blotting analysis of culture supernatant (a). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed after His-tag-purification of hEx3s (b). Each purified sample was added along with T-LAK cells to TFK-1 cells (T-LAK:TFK-1 ratio, 5:1) (c). The value of ta2 at 0.1 pM was not evaluated correctly due to technical error.

Evaluation of bsAbs expressed in B. choshinensis

Unlike *E. coli*, *B. choshinensis* is a highly safe Gram-positive bacterium that can secrete soluble recombinant proteins directly into culture. Therefore, we used *B. choshinensis* as an expression host and evaluated the function of bsAbs prepared from this expression system. All the bsAbs, except sc7, were successfully expressed and secreted in the culture supernatant. The levels estimated by western blot analysis of the culture

supernatant also revealed differences in the pattern of expression levels in *B. choshinensis* compared to *E. coli*. In *B. choshinensis*, sc1, sc3, and ta2, ta4, ta5 showed relatively high expression levels (Figure 4a). SDS-PAGE analysis also showed that purified His-tagged bsAbs prepared from *B. choshinensis* culture supernatants still contained a considerable amount of impurities (Figure 4b). A growth inhibition assay was performed using the bsAbs concentration calculated from band intensity. To confirm whether impurities from host cells affected growth, we first performed an MTS assay using culture supernatant directly. The diluted supernatants did not profoundly enhance the activities of T-LAK cells, indicating that the impurities in the bsAb solutions prepared from *B. choshinensis* had limited effects on cytotoxicity (Supplementary Figure 1). High growth inhibitory effects were observed in sc1, sc3, sc5, sc8, ta1, ta2, and ta6, similar to those expressed in *P. pastoris*. This indicates that *B. choshinensis* is a potentially viable production host for next-generation therapeutic bsAbs (Figure 4c).

Evaluation of highly purified bsAbs expressed in three different host cells

To confirm whether the same variants expressed from *E. coli, P. pastoris*, and *B. choshinensis* had comparable activities, we purified and evaluated bsAbs from the three different expression systems. The multimeric form of small recombinant antibodies often results in higher activity due to avidity.[25–27] There was a substantial amount of impurities in the purified His-tagged bsAbs from *B. choshinensis* (Figure 4b). For further investigation, we chose ta6 because it showed high growth inhibitory activity and relatively high expression levels in all the expression systems. While several peaks caused by multimeric structures and impurities were found in ta6 from *E. coli* (Figure 5a,b), single peaks corresponding to the monomers were mainly observed in ta6 from *P. pastoris* (Figure 5c, d) and *B. choshinensis* (Figure 5e,f). The final yields for monomers of ta6 from *E. coli, P. pastoris*, and *B. choshinensis* were approximately 10, 50, and 100 μg/L culture, respectively. The monomer fractions following His-tag purification were used for cancer growth inhibition assay, and no major differences were observed among the monomers of ta6 prepared using the different expression hosts (Figure 5g). These results show that expression levels vary depending on domain rearrangements and host species, but growth inhibitory effects of bsAbs are independent of the expression host used, at least as far as the representative microorganisms (*E. coli, P. pastoris*, and *B. choshinensis*) we tested in this study are concerned.

Discussion

A variety of expression systems for the production of recombinant antibodies such as bacteria, yeasts, filamentous fungi, insect cells, and plant cells have been explored. Although eukaryotic and prokaryotic expression systems have concerns regarding the immunogenicity of non-human glycosylation patterns and functional depression due to a lack of glycosylation,[28] both systems can be applied for small recombinant antibodies because many of them lack the Fc region that contains conserved functional glycosylation sites. *B. choshinensis* is a Gram-positive bacterium that secretes proteins directly into culture, has low extracellular protease activity, produces no endotoxins, and is nonpathogenic.[16–18] It is an attractive protein expression host, but it has never been used as a production host for small recombinant bsAbs.

Among small recombinant bsAbs, the Dbs and taFvs formats are the most widely used, and their features and structural differences have been reviewed.[11,29,30] The bsDb format has four possible configurations with different domain orders, and we previously reported that domain rearrangement affected the expression levels and cancer growth inhibition effects of hEx3-Db.[23] In contrast, both single-chain formats, scDb and taFv, have eight possible configurations, and effects of rearranging domain orders on either expression level or function have not been reported. Thus, in this study, we constructed all 16 configurations of small bsAbs based on hEx3 and determined their optimal host cells considering both expression levels and function.

Our comprehensive investigations on hEx3 formats using optimized whole genes showed that the expression levels varied depending on the bsAb formats and microbial hosts (Figures 2a,b, 3a, 4a), while variation in cancer growth inhibition effects was solely format-dependent (Figures 2c, 3c, 4c). When we used highly purified samples, it was confirmed that the intrinsic functions of hEx3 were similar to each other regardless of host cells (Figure 5g). In bsDb formats, we previously reported that LH type, in which both components are in the VL-VH order, exerted the strongest anti-tumor activity *in vitro* and *in vivo* among the four possible configurations.[23,31] We considered that the structure of LH type could avoid steric hindrance with cell surface molecules, and this feature might contribute to the intense cytotoxicity. In this study, sc1 and sc3 reflect the LH type single-chain formats. Among eight possible configurations for bispecific scDb, these LH type configurations also showed relatively high inhibitory effects, and we could not find other configurations with obvious significant effects other than them (Figure 3c).

No comprehensive investigations on the effects of configuration of bispecific taFv formats on antibody function have previously been reported. In our study, taFvs prepared using *P. pastoris* showed relatively high purities and high activities for ta1, ta2, ta6, and ta8, almost consistent with the results observed for other expression systems (Figure 3c). An important structural feature of the taFv format is its high flexibility because of the two binding sites that can rotate freely and allow it to facilitate the simultaneous binding of two antigen epitopes.[11] Our results showed that, despite the high structural flexibility of the taFv format, the cytotoxic activity of the antibody varied depending on the domain order. This suggests that each taFv variant also has different ranges of mobility and permissive angles for crosslinking two target cells, influencing cytotoxic properties.

B. choshinensis is an attractive candidate as a host cell for the production of next-generation biologicals, including small bsAbs. We optimized the culture conditions for expressing 16 hEx3 bsAb variants in *B. choshinensis*. As shown in Figure 6,

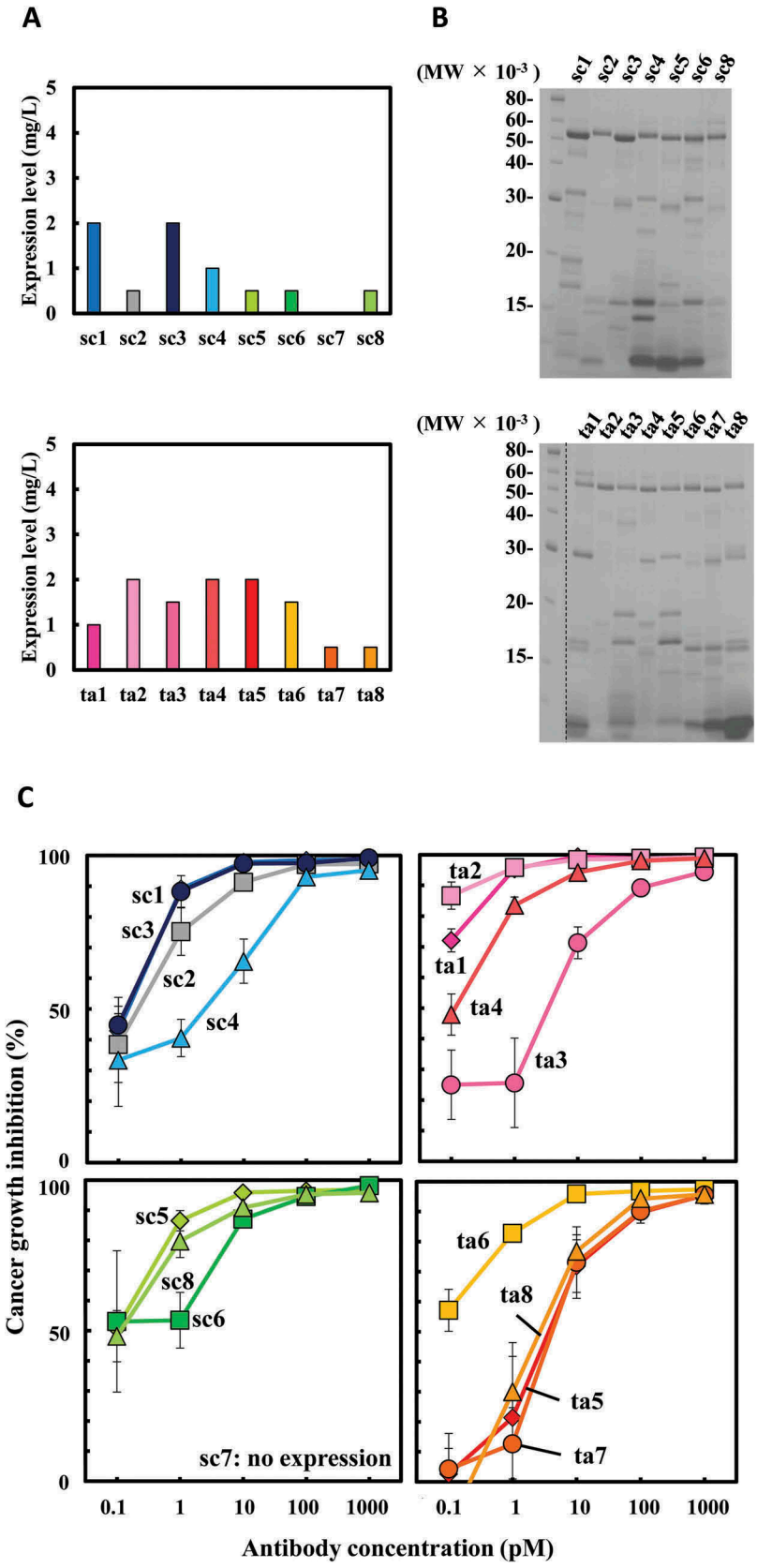

Figure 4. Expression of 16 hEx3 variants in *Brevibacillus choshinensis* and their growth inhibition effects against epidermal growth factor receptor (EGFR)-positive TFK-1 cells. Expression levels were estimated by western blotting analysis of culture supernatant (a). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed after His-tag-purification of hEx3s (b). Each His-tag-purified sample was added along with T-LAK cells to TFK-1 cells (T-LAK:TFK-1 ratio, 5:1) (c).

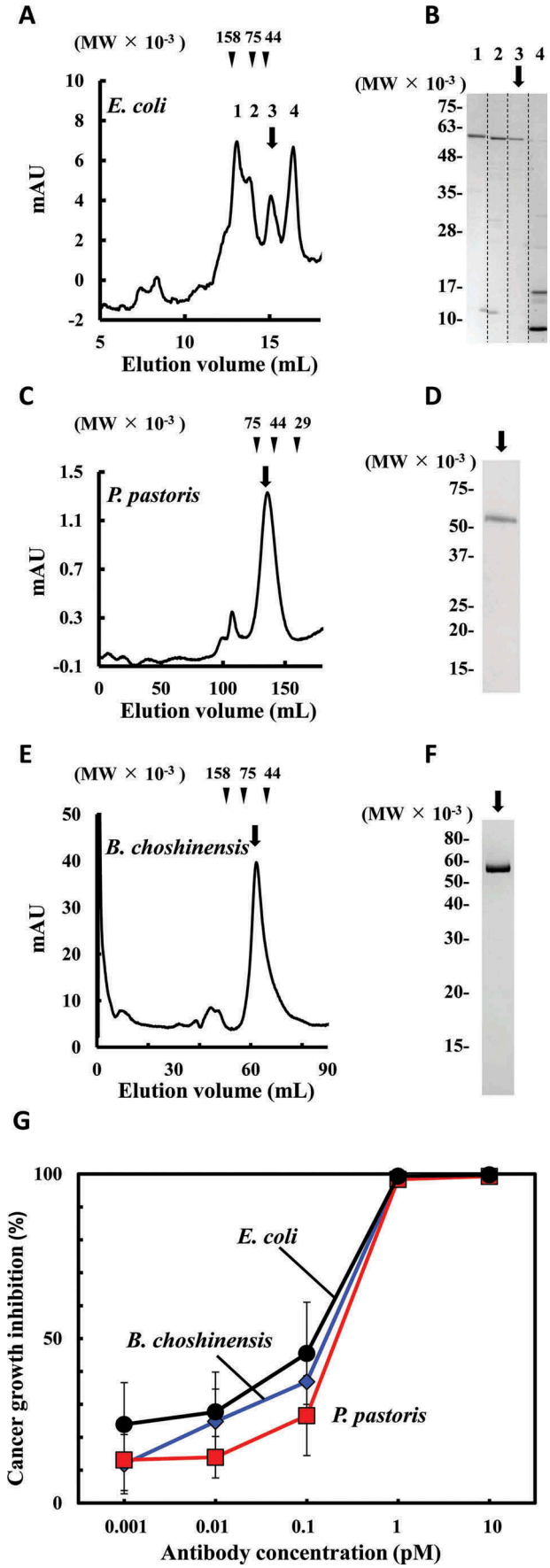

Figure 5. Comparison of growth inhibition effects against epidermal growth factor receptor (EGFR)-positive TFK-1 cells using the ta6 monomers prepared from three different host cells. A chromatograph and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for each peak after gel filtration of ta6 expressed in *Escherichia coli, Pichia pastoris*, and *Brevibacillus choshinensis* are shown (a, b, c, d, e, f). Each ta6 monomer indicated in arrow was added along with T-LAK cells to TFK-1 cells (T-LAK:TFK-1 ratio, 5:1) (g).

yields greater than 10 mg/L were observed in several variants upon addition of 0.05–0.2 M L-arginine for each variant, with ta6 expressing one of the highest yields (15 mg/L), as well as one of the highest cytotoxic effects among the 16 variants (Figure 3c). Similar expression levels were also confirmed in three different ta6-expressing *B. choshinensis* clones by western blotting (Supplementary Figure 2). Several examples of recombinant proteins, including antibody fragments, being produced in the g/L range in *B. choshinensis* have been reported.[32–34] A host-vector system using genetically engineered *B. choshinensis* HPD31-SP3 strain that cannot form spores nor secrete proteases is commercially available. Furthermore, in this system, three promoters with different expression activities can be combined with four signal sequences to optimize recombinant protein production.[32] We are currently working on the development and optimization of the fed-batch culture for promising variants such as ta6, considering vector constructions. Since the final yields (i.e., purification efficiencies) are still low, we are also developing an efficient downstream process.

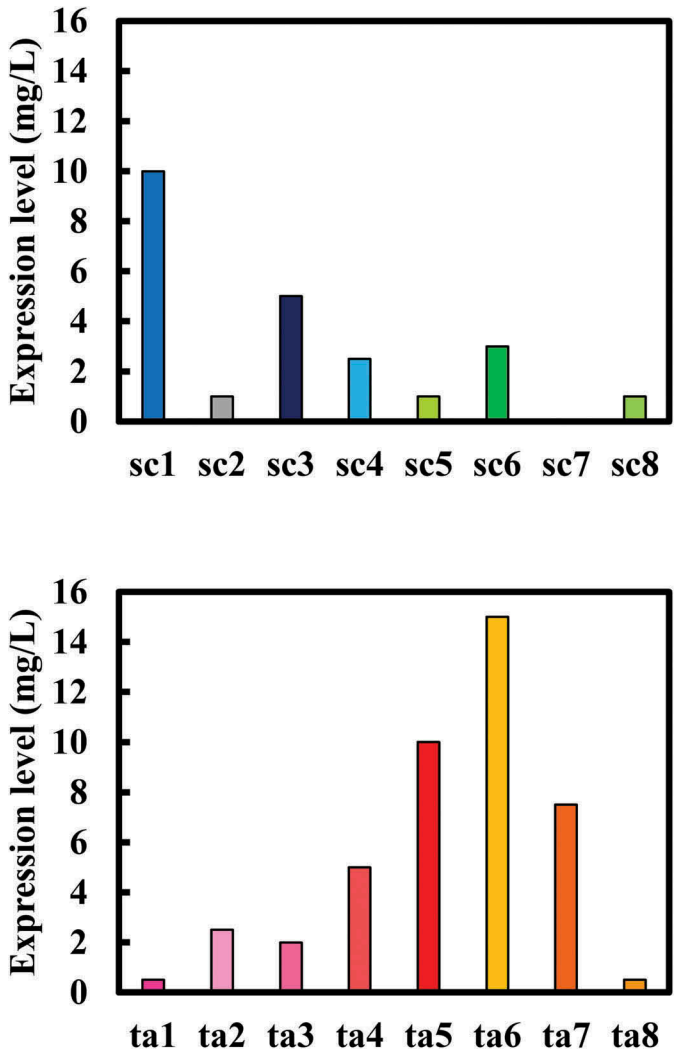

Figure 6. Optimization of expression by investigating the culture conditions of *Brevibacillus choshinensis*. Expression levels were estimated by western blotting analysis of culture supernatant.

In this study, we report the first evaluation of all configurations of bispecific scDb and taFv comprising identical Fv pairs. Comprehensive investigations showed that the intrinsic functions of hEx3 in each format were similar to each other, regardless of host cell, but expression levels varied depending on the format and host cell. Among the 16 hEx3 bsAb variants, we found a promising candidate that exhibited high activity and productivity. Moreover, *B. choshinensis* is an attractive microbial host cell for recombinant protein expression because of its secretory production of recombinant proteins.

Materials and methods

Construction of expression vectors for bsAbs with different domain orders

As in our previous report,[22] we designated here the VH and VL regions of the humanized anti-EGFR antibody 528 as h5H and h5L, and those of the humanized anti-CD3 antibody OKT3 as hOH and hOL. The high affinity mutant HY52W, in which Tyr^{52} was replaced by Trp, was used as h5H. We previously isolated this mutant using a phage display system.[35] We first designed all possible domain orders for single-chain bsAb format of hEx3, including eight types of hEx3-scDb and eight types of hEx3-taFv, as shown in Figure 1. To reduce linker-inherent effects on the function of bsAbs, we chose the standard simple glycine-rich linkers. Genes of variable regions optimized for *E. coli* codons and for *P. pastoris* codons were synthesized and used to construct expression vectors of 16 kinds of hEx3 bsAbs for each of the expression host species. The pRA vector, which is a previously constructed T7 promoter-based expression vector with N-terminus pel-B signal peptide and C-terminus Myc and His tags,[36] was used for *E. coli* expression system. The gene cassettes with the N-terminus α-factor secretion signal and C-terminus Myc and His tags were amplified using polymerase chain reaction (PCR) and were inserted into the pJ902-15 vector for expression in *P. pastoris*.[37] Because of the similarity in codon usage of *E. coli* and *B. choshinensis*, genes optimized for *E. coli* codons were inserted into the pROXb3 vector with N-terminus R2L6 signal peptide and C-terminus Myc and His tags for expression in *B. choshinensis*.

Expression of bsAbs using three host cells

Three host cells (*E. coli, P. pastoris*, and *B. choshinensis*) were used for the expression of 16 hEx3 variants. *E. coli* strain BL21 Star (DE3) (Life Technologies) was transformed with the pRA vectors and cultured according to the methodology described in a previous report.[38] *P. pastoris* strain PPS-9010 (ATUM) was transformed with the pJ902-15 vectors via electroporation after linearizing with SacI and cultured in buffered minimal glycerol-complex (BMGY) medium (pH 6; 2% peptone, 1% yeast extract, 1% glycerol, 1.34% yeast nitrogen base with ammonium sulfate but without amino acids, and 0.00004% biotin in 100 mM potassium phosphate buffer) at 30°C with shaking at 200 rpm until the optical density at 600 nm (OD_{600}) reached 20–50. To induce protein expression, the cells were resuspended in BMMY (same as BMGY, except

0.5% methanol is replaced with glycerol) to an OD_{600} of 10 and were cultured at 25°C for 48 h. The medium was re-supplemented with methanol every 12 h. *B. choshinensis* HPD31 strain[16] was transformed by electroporation and cultured at 30°C for 48 h in the medium with 4% Phytone™ peptone (BD Biosciences), 0.5% yeast extract, 0.001% $FeSO_47H_2O$, 0.0001% $ZnSO_47H_2O$, 0.001% $MnSO_45H_2O$, and 2% glucose.[39] Each supernatant was used for western blotting using horseradish peroxidase (HRP)-conjugated anti-His tag antibody to estimate the expression levels using Multitope (Denatured, recombinant, Solution, FUJIFILM Wako Pure Chemical Corporation) as a calibration marker protein.

Purification of bsAbs expressed in three host cells

E. coli transformants were harvested and sonicated. The intracellular soluble fraction was loaded onto a HisTrap HP column (GE Healthcare Bio-Science), and the eluted fraction was loaded onto a Superdex 200 GL column (10/300; GE Healthcare) to fractionate the monomers. Culture supernatants of *P. pastoris* transformants were loaded onto a TALON spin column (Takara Bio USA, Inc.) and a HiLoad Superdex 200 pg column (26/600; GE Healthcare) to fractionate the monomers. In the case of the *B. choshinensis* expression system, purification and fractionation of the monomers for bsAb were performed using a HisTrap HP column and a HiLoad Superdex 200 pg column (26/600), respectively.

Cell lines

Human bile duct carcinoma (TFK-1) was used in this study. The TFK-1 cell line was established by our group.[40] The TFK-1 cell line was cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

In vitro killing assay

Lymphokine-activated killer cells with the T-cell phenotype (T-LAK) were induced as described in literature.[41] Peripheral blood mononuclear cells were cultured for 48 h at a density of 1×10^6 cells/mL in medium supplemented with 100 IU/mL recombinant human interleukin 2 (IL-2) (Shionogi Pharmaceutical Co.) in a culture flask (A/S Nunc) that was pre-coated with anti-CD3 monoclonal antibody (10 μg/mL). The *in vitro* growth inhibition of cancer cells was assayed using an MTS assay kit (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay; Promega), as reported in literature.[41]

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was reported by the authors.

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