Biomedicals from a soil bug
Expanding scFv production host range

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Recombinant antibody fragments have a wide range of applications from research to diagnostics and therapy. Of special interest are small fragments like fragment antigen binding (Fab) or single chain fragment variables (scFv) fragments as they can be produced inexpensively in bacterial expression systems. However, recombinant production efficiencies from established production hosts vary significantly leading to inadequate yields. Gene sequences that have been synthetically adapted to match the codon preferences and respective genomic tRNA pool of the host have been used to improve yields but cannot resolve the principal problem. The development of inducible broad host range scFv expression plasmid constructs leads the way to an easy and efficient screening method for the identification of the optimal bacterial expression host.

Introduction

The demand for recombinant antibodies is increasing due to their broad applications from diagnostics to therapy; a fact clearly demonstrated by the growing marked for antibody based drugs with more and more drugs in this class progressing through clinical trials. Many of those exploit full-length antibodies produced in eukaryotic hosts to yield human or human like glycosylations. However, where effector functions are not necessary, minimized phage display derived modules like single-chain fragment variables (scFv) or fragment antigen binding (Fab or scFab), which retain the antigen-binding specificity without the fragment crystallizable (Fc) element, are advantageous. From an ethical point of view the phage display approach can help to reduce the amount of animal experiments in medical research. Furthermore, because the DNA sequence encoding the antibody is available, the small fragments offer numerous ways for the engineering and modification of biophysical properties to suit their operation purpose and are eligible for production at low costs and fast production cycles in microbial systems. These advantages however, are impaired by the often poor levels of soluble antibody fragments in prokaryotic production systems and the necessity of time consuming improvement approaches like codon-optimization or excessive screening of culture and expression conditions.

Newly introduced inducible scFv expression plasmid constructs expand the established production host range. Phage display selected human scFvs directed against a human cancer marker protein and potential therapeutic target, Mucin1, an "acute-phase" inflammation indicator in the human blood, C-reactive protein and a murine anti-hen egg-white lysozyme model antibody, D1.3 were used to assess the functionality of those new constructs. A feature of the plasmids is the chassis that is based on the well-studied RK2 broad host range plasmid which therefore should allow replication, maintenance and expression of scFvs in the majority of Gram-negative bacteria. A quality that enable the generation of an expandable bacterial broad host range expression vector cloning system that facilitates exploration of new hosts and the fast determination of the optimal host for the production of challenging recombinant antibody fragments.
Assessment of a New scFv Production Host

The new expression tools were applied to explore the scFv production capacities and periplasmic translocation efficiencies of the Gram-negative bacterium *Pseudomonas putida* KT2440. *Pseudomonas putida* KT2440 is a metabolically versatile, ubiquitous soil bacterium with considerable potential in a broad range of diverse industrial and environmental applications. Besides its certification as a biosafety strain KT2440 is well known for good expression of heterologous genes \(^{17-20}\) and therefore a good candidate for a new scFv expression cell factory.

The constructs are designed to include the widely used *Erwinia carotovora* pelB-leader peptide\(^ {21}\) for targeted translocation of the scFv peptide chain to the periplasmic space at the translation start (Fig. 1). The signal peptide is fused to the coding sequence of the variable domain of the heavy chain (VH), a linker and the variable domain of the light chain (VL) followed by affinity tag coding sequences (Fig. 1). The transcriptional control is set under an IPTG inducible Ptac-like lac/trp hybride promoter. These broad host range constructs can be introduced by a fast and convenient transformation into *Pseudomonas putida* KT2440 cells where they can be stably maintained by selection for the construct’s streptomycin resistance. At cultivation conditions and induction time points determined in optimization experiments (30°C for 180–200 min.) cells were harvested and lysed for scFv extraction. A fast single-step affinity chromatography purification using the Strep-tag\(^ {1}\)II’s affinity toward Strep-tacin sepharose yields soluble scFvs of good purity (1.5 mg/l for D1.3, 2.9 mg/l for anti-CRP and 3.6 mg/l for anti-MUC1, Fig. 2). The activity of the one-step purified anti-CRP and anti-MUC1 scFvs was assayed in ELISA-plates coated with CRP and a 32 amino acid MUC1 peptide. Antigen binding scFvs were visualized using a primary anti-Myc-tag antibody and a secondary HRP conjugated antibody giving a signal down to scFv concentrations of around 10 nM. The yields from *P. putida* KT2440 were 2.5–4-fold higher to analog experiments employing the identical constructs in *E. coli* background. The *E. coli* yields lie in the same area as those of previous experiments that produced 0.55 mg/l for an anti-CRP scFv\(^ {13}\) and 0.29 mg/l for the D1.3 scFv.\(^ {6}\) Best results were achieved for the IPTG inducible promoter which were more than twice those obtained by constructs where the Ptac/lacI\(^ {18,22}\) is replaced by a Pm/xyIs promoter/regulator gene system.\(^ {23}\)

The genome sequence of *Pseudomonas putida* KT2440 is characterized by a high average GC content (~61.5%). The impact of the scFv coding sequence GC-content and codon usage on production efficiencies was investigated using synthetic codon adapted sequences designed for anti-CRP and anti-MUC1 scFv genes. Remarkably, protein yields did not increase if synthetic gene sequences were used that have been codon usage adapted and enriched in GC content from wildtype 55.0/57.8% to synthetic 68.3/67.6% respectively (Fig. 2). Interestingly highly conserved translational machinery genes of *Pseudomonas putida* KT2440 do not show the high GC content.\(^ {19}\) This differential usage of t-RNA species for ribosomal proteins probably accounts for the metabolic versatility of *P. putida* KT2440\(^ {24}\) and in turn might explain the good expression levels achieved for scFv genes with various GC contents.

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**Figure 1.** Architecture and design of scFvs and expression constructs. (A) schematic representation of an scFv in the form of an antibody variable domain of the heavy chain (VH)-linker-variable domain of the light chain (VL). (B) crystal structure of murine D1.3 anti-hen egg-white lysozyme VH (blue) and V kappa (VL, light blue). (C) The translated part of the antibody expression constructs starts with the 22 amino acids periplasmic transport signal peptide pelB, followed by VH, a 12–15 amino acid linker, VL and peptide tags for detection/purification in the sequence Myc-tag/His6-tag/Strep-tag\(^ {1}\)II or His6/Myc-tag/Strep-tag\(^ {1}\)II. (B is generated from pdb1VFA\(^ {30}\)).
summary, *P. putida* KT2440 allows for the expression of heterologous genes with a broad spectrum of GC contents (and codon usages) and codon adaptation is not a prerequisite for high expression levels.

**Targeted Translocation to the Periplasma of *Pseudomonas putida* KT2440**

Whenever possible the secretion of a recombinant protein to the periplasma or the extracellular medium is advantageous because secreted proteins are more easily accessible, less contaminated with cellular proteins and usually have authentic N-termini. Often, folding is improved and proteolysis is less extensive compared with cytoplasmic productions. The secretion capacity is therefore an important trait of viable production hosts. The scFv constructs are expressed N-terminally extended by the pelB-leader sequence.21 Periplasmic extractions and purifications were performed and compared with whole cell extraction purifications that include both cytoplasm and periplasm localized proteins. This enables control of completeness of transport and signal peptide cleavage by protein gel blot analyses detecting the C-terminal affinity tags. Applied to the chosen scFv system unprocessed protein was only detectable in anti-MUC1 purifications from whole cell extractions, but neither in purifications of anti-CRP fragment production nor periplasmic preparations. This demonstrates that all translocated protein is processed by cleavage of the pelB-leader sequence upon transport. In one case anti-CRP fragment yield was higher than anti-MUC1 yield (Fig. 2). An overloading of the secretion apparatus can therefore be excluded as a reason for the observed unprocessed anti-MUC1 in this experiment. It seems rather likely that the sequence following the pelB leader sequence negatively influences transport and processing or gave rise to the production of insoluble anti-MUC1 in this experiment. The exact cleavage position as predicted by bioinformatics22 was verified by N-terminal sequencing of the purified scFvs revealing the precise cleavage after amino acid 22, corresponding to the signal peptidase I recognition motif AXA (AMA).

**Conclusion**

Recombinant antibody fragments that bind to antigens of high biomedical interest for diagnostics and therapy are employed to evaluate *Pseudomonas putida* KT2440 as a protein factory. The engineered expression allowed the production of soluble and active scFvs at higher yields as compared with *E. coli* expression experiments. Notably the highest expression was achieved with the native non-codon adapted sequences. Good expression yields could be achieved using scFv gene sequences, spanning GC content differences of 16%. The targeting of the recombinant protein to the periplasmic space of *P. putida* KT2440 worked well making use of the heterologous pelB leader sequence that was cleaved precisely and completely upon transport. Thus, periplasmic production might be a good alternative also for the expression of moderately toxic proteins with this system. A single step affinity purification procedure via a C-terminal Strep-tagI enabled the fast and simple recovery of scFvs especially from the periplasmic extract. The new
constructs herewith expand the established scFv production hosts by a versatile soil microbe. By far not all possibilities for optimization are exploited. A simple test of different media indicated the potential for further improvement by use of more enriched and buffered media. The presented results yield from shake flask batch culture experiments with moderate cell densities compared with the very high biomass concentrations that have been achieved for Pseudomonas in bioreactors.

The culture conditions should therefore comprise enormous potential to multiply the product quantity per culture volume. Besides, there are numerous possibilities for synthetic engineering and streamlining of a microbe to further improve its production properties.

**Perspective**

The broad host range chassis of the presented new constructs will allow the construction maintenance and expression of recombinant antibody fragments or other proteins in several hosts other than *Pseudomonas putida*. The ability to screen expression hosts and explore suitable hosts for individual proteins may help to overcome the marked demand for recombinant antibodies by enhancing the number and quality of proteins that can be produced at the fast production cycles and low costs given by microbial systems. This is of particular importance from different points of view: on one side cost-effective protein expression and purification strategies are required not only to produce the antibodies itself but also to produce target proteins that are a prerequisite for antibody generation and screening. Today several projects aim at using the available genome information to generate antibodies against the whole human proteome to gain new insights into the functions of the gene products. Recently the availability of an almost complete human anti-proteome may in future lead to a more personalized medicine. On the other hand there is a huge unexploited capacity of newly isolated and described bacteria of which many are already genome sequenced. The example described herein in which a promising expression host for biomedicals was found by applying a flexible expression tool to a well-known host that was previously not explored for this purpose should motivate screening of the available phylogenetic diversity for biomedical and biotechnological protein production purposes.

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