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Enhancing the Solubility of Recombinant Akt1 in *Escherichia coli* with an Artificial Transcription Factor Library

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Abstract A combinatorial library of artificial transcription factors (ATFs) was introduced into the bacterial cells that expressed the Akt1-GFP fusion protein. By measuring the level of fluorescence generated by the transformed *E. coli* cells, we were able to obtain clones in which ATFs increased the solubility of the Akt1. Our results show that ATF library is a useful tool for increasing the solubility of selected recombinant proteins in *E. coli.*

Key words: Recombinant proteins, solubility, artificial transcription factors

Because of its rapid growth and rate of protein production, combined with a plethora of advanced genetic tools and volumes of information on its physiological properties, *Escherichia coli* is currently the most frequently used prokaryotic expression system for high-level production of heterologous proteins [9, 11, 14]. However, overexpressed heterologous proteins, especially from those of eukaryotes, often accumulate in *E. coli* cells as insoluble inclusion bodies [20, 21]. Although production of a recombinant protein as insoluble aggregates offers an advantage of easy purification, a protein that has been solubilized by some appropriate process, such as urea treatment, might not retain the activity of the native protein.

Current approaches to improve the expression of soluble recombinant proteins in *E. coli* include fusion of the protein of interest with a more soluble partner protein [6, 7, 10, 22], coexpression of folding catalysts and chaperones [1, 17, 25], and growth of the bacteria at a reduced temperature or in a modified medium [15, 17]. However, the success of these approaches in terms of their ability to confer solubility upon heterologous proteins is unpredictable, particularly when the host cells are cultured at optimum growth temperatures. Optimization of conditions for the production of a heterologous protein in a soluble form can be a time-consuming and labor-intensive process.

Zinc finger-containing proteins represent a large and diverse class of transcriptional regulatory proteins. Each zinc finger domain consists of about 30 amino acid residues, which usually binds in a sequence-specific manner to 3 base pairs (bp) of DNA. One common version of the zinc finger domain is the Cys_2-His_2 finger. Because of the modular nature of zinc fingers, artificial transcription factors (ATFs) have been constructed by fusing three or four zinc finger domains (ZFDs) together to form multi-finger proteins that recognize specific 9-bp or 12-bp DNA sequence elements, respectively [4, 13, 24]. The fused ZFDs can serve as gene-specific transcriptional repressors on their own, or can be fused with a transcriptional activation or repression domain. Several studies report that zinc-finger-containing ATFs can regulate the expression of a variety of genes in mammalian, plant, and bacterial cells [3, 8, 18, 19].

It is now possible to construct randomized ATF libraries that consist of tens of thousands of distinct, active transcription factors [5, 16]. ATF libraries have been used to induce phenotypes of interest in transformed cells, such as resistance to heat or osmotic shock in *E. coli* or yeast [13] and cell differentiation, cell proliferation, or resistance to taxol in mammalian cells [16]. Thus, it is plausible that ATF libraries can be used to produce phenotypes in *E. coli* cells that increase the solubility of recombinant proteins.

Recently, it was reported that inclusion body formation by recombinant proteins fused to green fluorescent protein (GFP) prevents proper GFP folding, and thus formation of the GFP fluorophore [23]. In contrast, successful folding of fusion proteins results in the accumulation of fluorescent GFP. In addition, it has been shown that the amount of fluorescence in bacterial cells expressing a GFP fusion protein is directly related to the amount of recombinant protein found in the supernatants of lysed

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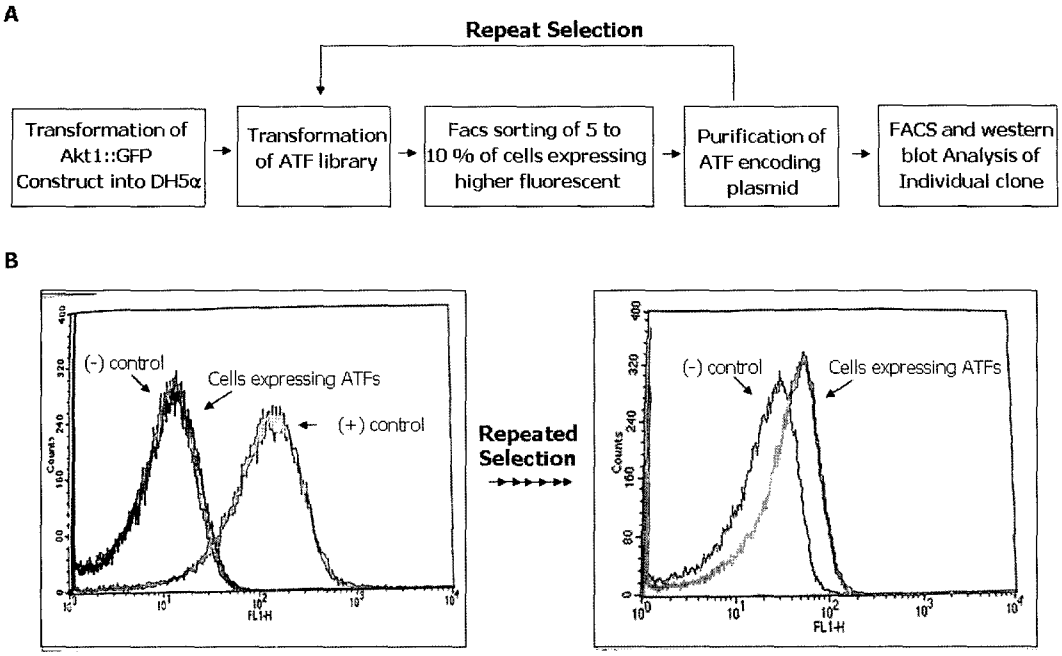

Fig. 1. A. ATF library screening procedure for ATFs that increase the solubility of the human Akt1 recombinant protein in *E. coli*. The Akt1-GFP fusion construct was transformed into *E. coli* strain DH5α. These transformed cells were then retransformed with an ATF library, and the doubly-transformed cells that displayed enhanced fluorescence were selected by FACS analysis (5 to 10% of the transformed cells were selected). An ATF-encoding plasmid was purified from each of the selected cells and retransformed into an Akt1-expressing cell. The whole procedure was repeated eight times, and two ATFs were finally selected. These ATFs were then analyzed by FACS and Western blotting for their ability to increase the solubility of Akt1. **B.** Comparison of the amount of fluorescence displayed by *E. coli* cells collected after the first and eighth rounds of selection. First selection: (-) control cells (untransformed), green; ATF-expressing cells, blue. Eighth selection: (-) control cells (untransformed), blue; ATF-expressing cells, red.

bacterial cells that expressed the corresponding nonfusion protein under identical conditions.

In this study, using a GFP-containing recombinant protein as a reporter, we show that an ATF library can be applied to increase the solubility of recombinant protein kinase B (Akt1) in *E. coli*.

We measured the whole-cell fluorescence of *E. coli* cells expressing Akt1 as a GFP fusion protein. The cDNA of Akt1 was cloned into the pET21b vector, and GFP was ligated to the C-terminus of the Akt1 open reading frame. Most of the Akt1 recombinant protein was produced as an insoluble form (data not shown). As expected, GFP fused with Akt1 was not fluorescent. The GFP used in this study was folded well when expressed alone in *E. coli*; therefore, this condition served as a positive control.

The ATF library for *E. coli* was constructed by subcloning DNA that encoded 4-finger ATFs into pZL1, which is an expression vector for *E. coli* [16]. Figure 1A outlines the procedure for screening of the ATF library to select ATFs that increased the solubility of the Akt1 recombinant protein in *E. coli*. *E. coli* strain DH5α, which expressed the Akt1-GFP fusion protein, was transformed with the ATF library. An overnight culture of the transformants was diluted to 1:500 in 1 ml of fresh Luria broth (LB) containing 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to induce ATF expression. After 3 hours of incubation at 30°C, 10^6 cells were analyzed with the FACS Vantage flow cytometer cell sorter, and cells (5 to 10%) that expressed higher amounts of fluorescence were collected. The plasmid DNAs that encoded ATFs were isolated from the selected cells and used to retransform *E. coli* cells that expressed the Akt-GFP fusion protein. The same selection procedure was repeated eight times to enrich ATFs that increase the solubility of Akt1-GFP. Cells transformed with pZL1, which is an empty vector used for the ATF library constructs, were used as a negative control to set the background of the FACS analysis.

From this series of experiments, we determined that the increase in the fluorescence of *E. coli* cells was proportional to the number of enrichment cycles (data not shown). The ATF-expressing *E. coli* cells showed a markedly increased fluorescence after the eighth round of the FACS analysis (Fig. 1B). Therefore, after the eighth cycle, ATF-encoding plasmids were isolated from the highly fluorescent sorted cells, and the identity of these ATFs was determined by nucleotide sequencing. Two ATFs, ATF-S1 and ATF-S2, constituted the major AFTs in the enriched sample, and they were finally selected for further analysis.

E. coli cells transformed with ATF-S1 or ATF-S2 expression plasmids were shown to be more fluorescent than control cells that had been transformed with pZL1

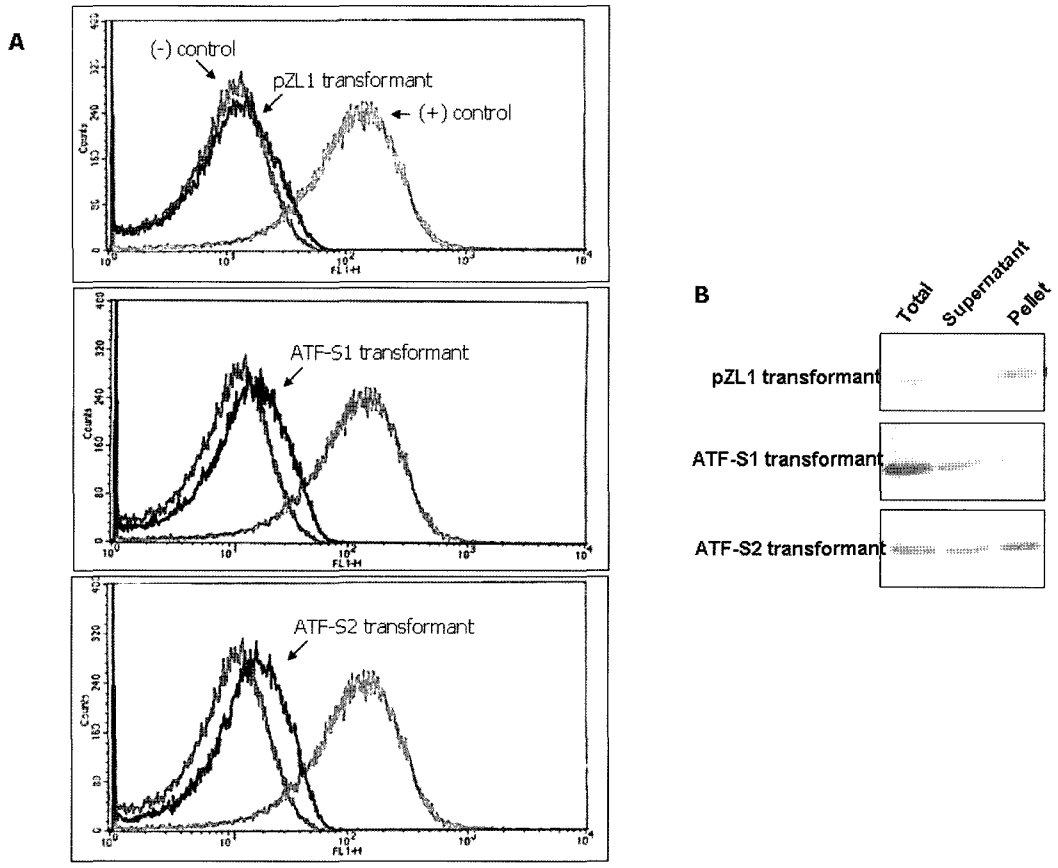

Fig. 2. Increased solubility of recombinant Akt1 by ATF-S1 and ATF-S2 analyzed by FACS (A) and Western blotting (B).
pZL1 is a backbone plasmid that was used for construction of the ATF library for *E. coli*. **A**. Top panel: (-) control cells (untransformed), green; pZL1 transformant, blue; (+) control (GFP-expressing cells), red. Middle panel: (-) control cells (untransformed), green; ATF-S1 transformant, blue; (+) control, red. Bottom panel: (-) control cells (untransformed), green; ATF-S2 transformant, blue; (+) control, red. **B**. Cell extracts from ATF-S1, ATF-S2, and pZL1 transformants were stained with anti-Akt1 antibodies to quantify the Akt1 protein in the soluble and insoluble fractions of total cell extracts.

(Fig. 2A). Western blotting revealed that the amount of soluble Akt1-GFP that was purified from ATF-S1 or ATF-S2 transformants was markedly increased, compared with that of control cells (pZL1 transformants) (Fig. 2B). The result of the Western blot was quantified with QUANTITY ONE image analysis software (*Bio-Rad*, Hercules, CA, U.S.A.). Cells transformed with ATF-S1 produced twice as much soluble Akt1-GFP recombinant protein compared with the control (Table 1).

In summary, we present a method that uses a randomized ATF library to increase the solubility of recombinant proteins in *E. coli*. Because selected ATFs are transferable, it is possible to simultaneously express several ATFs in one cell to regulate multiple target genes involved in the solubility mechanism. When used in combination with other current technologies, such as genome-scale gene expression analysis, the ATF library method could also be useful for identifying genes and defining physiological

Table 1. Quantitative analysis of the activity of ATF-S1 and ATF-S2 in the production of soluble recombinant Akt1 protein in *E. coli*.

Transformed plasmid	ZFDs (N- to C- terminal)[a]				Yield of soluble Akt1 (%)[b]
ATF-S1	QSTR	DSAR	RDHT	WSNR	47.1±7.5
ATF-S2	VSTR	DGNV	QSNR	QSNK	45.7±6.2
pZL1[c]					21.5±1.8

[a]Each zinc finger domain was named using the single-letter abbreviations for the four amino acid residues at positions -1, 2, 3, and 6 in the alpha-helix of the zinc finger. These four residues are not contiguous and do not represent the entire amino acid sequences of the zinc finger domain. The entire amino acid sequences of the zinc finger domains used here were described previously [2].

[b]Values represent the percentage of soluble Akt1 in total amount of Akt1 protein produced; the numbers provided are mean values of two different determinations.

[c]pZL1 is a backbone plasmid that was used for construction of the ATF library for *E. coli*.

pathways [13, 16] involved in protein solubility in *E. coli*.

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