High-Throughput Screening of Acyl-CoA Thioesterase I Mutants Using a Fluid Array Platform

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ABSTRACT: Screening target microorganisms from a mutated recombinant library plays a crucial role in advancing synthetic biology and metabolic engineering. However, conventional screening tools have several limitations regarding throughput, cost, and labor. Here, we used the fluid array platform to conduct high-throughput screening (HTS) that identified *Escherichia coli* 'TesA' thioesterase mutants producing elevated yields of free fatty acids (FFAs) from a large (10⁶) mutant library. A growth-based screening method using a TetA-RFP fusion sensing mechanism and a reporter-based screening method using high-level FFA producing mutants were employed to identify these mutants via HTS. The platform was able to cover >95% of the mutation library, and it screened target cells from many arrays of the fluid array platform so that a post-analysis could be conducted by gas chromatography. The 'TesA' mutation of each isolated mutant showing improved FFA production in *E. coli* was characterized, and its enhanced FFA production capability was confirmed.

INTRODUCTION

Free fatty acids (FFAs) have attracted a lot of attention because of their potential as a controllable and renewable energy source.¹,² FFAs are also useful in the production of biofuels, cosmetics, lubricants/solvents, and pharmaceutical drugs. The corn-based production of FFAs is problematic because it competes with food production, increases refinery costs, decreases land-use efficiency, and raises environmental concerns. However, microbial-based FFA production shows potential in terms of its economic cost, quick production time, and smaller space requirements.³⁻⁶ Because *Escherichia coli* is a commonly used industrial organism, various approaches have been suggested that may improve the microbial production of FFAs.

Over the last decade, the overexpression of the rate-limiting enzyme and deregulation of the degradation pathway have been implemented to increase the microbial-based production capacity of FFAs and their precursors, leading to 21.5 g/L of FFA with 0.5 g/L/h productivity.⁷⁻¹² Microbial FFAs are mainly synthesized from acyl-ACP (acyl carrier protein) by the enzyme thioesterase I (TesA).³ The enzymatic characteristics of TesA have been thoroughly characterized; therefore, several previous studies have used it to increase the cellular production of FFAs.¹³⁻¹⁶ However, Jiang and Cronan¹⁷ reported that the overexpression of the enzymatic component in the fatty acid synthesis (FAS) pathway does not guarantee maximum FFA production in *E. coli*. Therefore, it is required to ensure optimal activity from key enzymes and components in the FAS pathway and subsequently improve production yields.

To find an alternate FFA production method that relieves the dependence on the excess expression level of 'TesA', which requires signal sequence-deficient *E. coli* acyl-CoA thioesterase I, we previously found a 'TesA' mutant using a conventional high-throughput screening (HTS) approach. We screened a 'TesA' mutant library to identify mutants that produce more FFAs than the wild-type (WT) enzyme.¹⁷ The isolation of desired mutants from a large mutant library is typically labor-intensive. Therefore, a two-step selection system was applied: (1) FFA-dependent tetracycline resistance for the growth-based enrichment of the desired mutants and then (2) selective isolation through FFA-dependent RFP expression with FACS-Calibur. However, only a few samples from the mutant library could be isolated during the growth-based enrichment phase because of competitive growth in the antibiotic's presence. Still, the growth of mutants that possibly display suboptimal FFA production can be affected by competitive crosstalk in the over-populated condition.¹⁸ Therefore, this screening method requires compartmentalization to avoid such crosstalk. Several microfluidic approaches have been implemented to overcome difficulties with compartmentalization and achieve complete coverage of large mutant libraries. These methods have several benefits when applied to HTS as an alternative to conventional experimental methods.¹⁹⁻²² Screening a library of more than 10^5 microbial mutants has never been achieved
using a microfluidic platform due to the trade-off between the compartmentalized environment of HTS and the difficulties associated with the selective isolation of desired mutants. We previously described a fluid array platform that had been specifically designed for the HTS of a large, microbial random mutation library. The simplicity of device fabrication and the ease of identifying extraordinary samples mean that the fluid array platform was successful in several different HTS applications that involved the growth-based and reporter-based screening of a microbial mutant library.

This current study combines the previously mentioned fluid array screening approach and two-step screening (cell enrichment in tetracycline-containing medium and RFP expression dependent on cellular FFA production). This resulted in the isolation of TesA mutants that display higher FFA production than WT TesA. We applied a hybrid screening procedure that used both reporter-based and growth-based screening simultaneously. In addition, we employed the fluid array platform against the random mutant library in an isolated microenvironmental culture condition to avoid the exclusion of suboptimal mutants. The selected TesA mutants were tested to confirm that cells expressing mutants produced higher amounts of FFAs than cells expressing WT TesA.

## EXPERIMENTAL METHODS

### Materials and Reagents.

The fluid array platform was fabricated with two types of oil with different specific gravities (SG) to compartmentalize the aqueous solutions: hydrocarbon oil (hexadecane, SG = 0.76, Sigma-Aldrich, Korea) and fluorinated oil (FC-40, SG = 1.61, Sigma-Aldrich, Korea). Cells were cultured in Luria Bertani broth (LB, Sigma-Aldrich, Korea) supplemented with antibiotics such as ampicillin (Amp, 75 μg/mL), chloramphenicol (Cm, 50 μg/mL), and tetracycline (Tet, 50 μg/mL) (all purchased from Bioshop Canada Inc.). An LB agar plate (1% w/v Agar, Becton Dickinson) was prepared for colony formation.

HTS with the fluid array used M9 minimal medium (Minimal salt 5X, Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂, 0.5% (w/v) glucose, an appropriate antibiotic concentration, and 0.3 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for the growth-based screening of the mutant library. Yeast extract 1 g/L (w/v) and trace elements were also added. The trace elements consisted of 2.4 g of FeCl₃·6H₂O, 0.3 g of CoCl₂·6H₂O, 0.15 g of CuCl₂·2H₂O, 0.3 g of ZnCl₂, 0.3 g of Na₂MoO₄·2H₂O, 0.075 g of H₃BO₃, and 0.495 g of MnCl₂·4H₂O per liter. The pH was maintained at 7.0 with 100 mM of sodium phosphate buffer (pH 7.0).

According to the previous report and product sheet of the fluorinated oil, the limit of solubility of fluorinated oil toward distilled water is 0.02% (v/v) at theoretical maximum. Therefore, to prevent the aqueous phase of the fluid array shrinking, FC-40 oil was mixed with distilled water (0.1% v/v) using a rotary shaker at 60 °C prior to the screening experiment. After the overnight stirring, the distilled water was not thoroughly mixed with the oils; thus, the final concentration of water in the oil was estimated to be approximately 0.02% (v/v). The distilled water saturated with fluorinated oil was used to prepare LB broth and M9 minimal medium for microbial experiments in this study.

### Preparation of the Bacterial Cells and GC Analysis of the FFAs.

E. coli MG1655 was the parental strain for all strains used in this research. The cell concentration was determined by measuring the optical density at a wavelength of 600 nm (OD₆₀₀) using a spectrophotometer (Libra S22, Biochrom Ltd, Cambridge, UK). For HTS, the TesA mutant library and fatty acid biosensor (FAB) cells harboring the biosensor plasmid pFAB were prepared as described previously.

GC analysis was conducted to analyze microbial FFA production in a precise, quantitative manner. The selected cells were cultivated in LB medium and diluted 1:100 in the previously described M9 minimal medium supplemented with 2% (w/v) glucose. When the OD₆₀₀ reached around 1.0, IPTG was added to reach a final concentration of 0.3 mM for the induction of TesA expression. After 48 h of incubation that resulted in microbial FFA production, 500 μL of each culture was stored at −20 °C until analysis. Next, 50 μL of 6 N HCl, 500 μL of ethyl acetate, and 50 μL of 1 g/L methyl nonadecanoate (Sigma-Aldrich) were added in sequence as an internal standard. FFAs were extracted from the cultures by vortexing for 30 s and centrifuging for 2 min. An additional 500 μL of ethyl acetate was added, and the same extraction procedure was repeated. Extracted FFAs were methylated by adding 100 μL of MeOH: 6 N HCl (9:1, v/v) and 100 μL of TMS-diazomethane (Sigma-Aldrich).

The FFA analysis was performed with a GC system (Agilent 7890A) equipped with a flame ionization detector and DB-5 column (30 m × 0.25 mm, Agilent Technologies). The FFA concentrations were measured by comparing each peak with the external standards composed of C₁₀-22 fatty acid methyl esters (Sigma-Aldrich). An oven temperature of 60 °C was maintained for 2.5 min, increased to 250 °C at 20 °C/min, and maintained for 4 min. Finally, the temperature was increased to 325 °C at a rate of 10 °C/min, which was maintained for 5 min.

### Statistical Modeling of the Library Coverage and Optimal Seeding Condition.

Two different genetic screening tools that are similar to a dual-selection system were simultaneously employed in this fluid array screening process to obtain mutants of TesA with increased functionality. In our previous research, the first screening tool was considered growth-based. It used the modified genetic circuit for the FFA-dependent tetracycline resistance gene (tetA) expression, which differentiates the cellular doubling time to enable the enrichment of cells that overproduce FFA. The second screening tool was reporter-based and employed an FFA-sensing reporter system. It showed a dose–response of fluorescence intensity caused by the presence of intracellular fatty acids. Optimal experimental conditions of FFA-dependent growth and RFP expression were previously investigated.

To adequately cover the mutant library, we constructed a statistical formula (presented below) derived from the Poisson distribution. According to eq 1, P is defined as the probability of covering the whole mutation library, f is the number of mutants in the library represented by collected colonies, and N is defined as the amount of coverage required to get P.

\[
N = \frac{\ln(1 - P)}{\ln\left(1 - \frac{1}{f}\right)}
\]

For the 10⁶-random mutation library of TesA, an experiment with an initial seeding number of 30 cells per chamber and 20 fluid array devices was carefully designed after considering the library coverage. Therefore, according to the above equation (eq 1), the experiment screened approximately
1.8 × 10^6 samples, which statistically covered >95% of the mutation library. Thirty cells were initially seeded and incubated for 20 h to ensure the differentiation and enrichment of cells whose growth was FFA-dependent. In addition, the incubation time enhanced the fluorescence signal from the FAB biosensor plasmid.

In terms of throughput, the 20 fluid array chips prepared and used in parallel covered >95% of the ‘TesA’ mutant library. It was assumed that 30 unique, initially seeded library cells resulted in the growth of only a few mutants because the enrichment from FFA-dependent tetracycline resistance was used as the first selective pressure. As a second screening method, we measured the fluorescence intensity in the fluid array device chambers generated by RFP, which corresponded to the intracellular FFA concentration in a quantitative manner. Samples with the highest fluorescence intensities (top 1%) were selected. The cells in the samples were isolated and named as UFM. The fatty acid production from at least five individual colonies per chamber was analyzed by GC.

**Fabrication of the Fluid Array Device.** The microwell in the fluid array device was 100 μm in diameter and 150 μm deep having 3000 arrays in a single device (3 × 3 cm). The spacing between individual arrays on a device was redesigned from 4 × 2 to 3 × 2 matrices based on the recovery efficiency of the glass capillary extraction process in our previous work. The SU-8 (Microchem 2075, Newton, MA, USA) master mold was fabricated using standard photolithography processes. The processed surface of a Si-wafer was silanized using trichloro-(3,3,3-trifluoropropyl) silane (Sigma-Aldrich, Korea) in a vacuum jar for 1 h. Polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI, USA) was then cast, cured, and peeled off to prepare the fluid array devices. To ensure that the environment was fully moisturized, the demolded PDMS devices were dipped in distilled water for several hours, resulting in a fully hydrated state (i.e., the highest solubility of water into PDMS). This ensured that the aqueous fluid in the microwell remained stable without apparent volume shrinkage over 24 h. During long-term incubation (>12 h), the fluid array device was half-dipped in oil and half-dipped in water to minimize array chamber drying.

**Experimental Setup and Data Analysis.** An inverted fluorescence microscope (IX-71, Olympus, Tokyo, Japan) equipped with a CCD camera (Clara, Andor, Belfast, Northern Ireland) and 1.5×, 10×, and 20× objective lenses was used to acquire all fluorescence images from the fluid array device. The images were automatically acquired using a microscope stage controller (MAC5000/Bioprecision2, Ludl Electronic Products, Hawthorne, NY, USA) with a multidimensional acquisition function from the Metamorph 7.1 (Molecular Devices, Sunnyvale, CA, USA) image software. The fluorescence intensities of the images were then performed with the high-throughput analysis using a custom-made m-file in MATLAB R2014a (Mathworks, Natick, MA, USA). Image J (NIH, Bethesda, MD, USA) and OriginPro 8 (OriginLab, Northampton, MA, USA) were used for additional data analysis and necessary image processing. A manually controlled probe positioner (PBS50, MS Tech, Hwaseong, Korea) was used to fix capillary tubes to extract target cells. Target cells were extracted with fused-silica capillary tubes (CAT no. #1068150011, Polymicro Technologies, Phoenix, AZ, USA) with 25.3 μm inner diameter and 360 μm outer diameter (OD) and commercially available insulin syringes with OD = 200 μm.

**Experimental Protocol for Screening.** The screening procedure was introduced in our previous work and is shown in Figure 1. The demolded PDMS fluid array devices were treated with oxygen plasma under 15 sccm of O2 and 70 W for 30 s (Cute-MP, Femto Science, Hwaseong, Korea). The mutation library in the medium (M9 minimal, SG: 1.0) with 0.3 mM of IPTG was deposited on the plasma-treated device and commercially available insulin syringes with OD = 200 μm. The Fluid Array Device was used as the cell medium on the device by the PDMS slab, the device was then filled with 20 Fluid array devices for screening of 10^6 library. (e) HTS strategy used to identify mutants with increased ‘TesA’ functionality. (a) Wild type ‘TesA’. (b) Introduction of mutation by error-prone PCR. (c) Construction of random mutant library. (d) Introduction of liquid array devices for screening of 10^6 library. (e) HTS strategy used to identify mutants with increased ‘TesA’ functionality. (a) Wild type ‘TesA’. (b) Introduction of mutation by error-prone PCR. (c) Construction of random mutant library. (d) Generation of fluid array devices for screening of 10^6 library. (e) HTS strategy used to identify mutants with increased ‘TesA’ functionality. (a) Wild type ‘TesA’. (b) Introduction of mutation by error-prone PCR. (c) Construction of random mutant library. (d) Generation of fluid array devices for screening of 10^6 library.
hydrocarbon oil (hexadecane, SG: 0.76) to retain the aqueous volume of the fluid array from the dehydration, so that top 1% of selective extractions of extraordinary mutants can be performed using glass capillary tubes for post-analysis of mutant cells. Because the recovery of the isolated cells was already optimized in the previous work,22 the isolated mutants were cultured in fresh medium. Finally, we identified the mutated region of ‘TesA’ of the isolated mutants for further investigation of the effects of mutations on FFA production.

■ RESULTS AND DISCUSSION

Isolation of Mutants Producing High Levels of FFA.
Several sensing systems were previously utilized to detect and quantify the production level of various biochemicals.26,27 To screen mutants overproducing FFAs in the ‘TesA’ mutant library, we used a previously constructed FFA biosensor composed of a TetA-RFP fusion protein under FadR-regulated promoter. In E. coli, the FadR acts as an acyl-CoA-responsive transcriptional regulator that binds DNA to inhibit the expression of several genes involved in fatty acid degradation pathway.28 As FFAs can be converted into acyl-CoAs, the intracellular concentration of acyl-CoAs is well correlated with FFAs in E. coli.28 In the FAB, high level of acyl-CoAs releases of the protein from its DNA-binding sites and increases the expression of TetA-RFP under a FadR-repressed promoter, resulting in high tetracycline resistance and red fluorescence.16 This FFA biosensor was employed to selectively isolate highly functional ‘TesA’ mutants.16,29 For the screening approach, the fluid array platform exploited growth-based screening using FFA-dependent tetracycline resistance during enrichment and reporter-based screening using FFA-dependent RFP expression. This study used this combined screening approach to integrate two genetic screening tools into a one-step screening approach. As previously discussed, we determined that using 30 individual mutant cells per chamber was appropriate for the FFA-dependent cell growth in medium containing tetracycline to cover the 10^6 mutant library. After incubation in the fluid array platforms and data analysis, the capillary extraction of cells in the chamber was conducted. Each cell broth was extracted in descending order of fluorescence signals produced from each chamber depending on RFP expression (Figure 1). Finally, 18 samples (UFM 1–18) were chosen based on the hybrid screening strategy, and their FFA production were measured by GC analysis for (i) the mutants selectively extracted by order of fluorescence intensity and (ii) the randomly extracted 20 mutants.
increased FFA production according to the results of GC analysis.

To examine possible correlations between the screening performance of the fluid array and FFA production levels, the selected mutants were categorized into two separate groups. The first group consisted of 18 mutants collected from the top 1% of the chambers that showed the highest fluorescence intensities from the fluid array device, and another group of 20 randomly selected samples was used as a comparison group. The average titer of FFAs from the first group was significantly higher than that of the second group (Figure 2). The results of the first group with the higher titer of FFA validated the screening approach used in this study.

**Genetic Identification of the Isolated Mutants.** DNA sequencing was conducted to characterize the 'TesA mutations in each mutant as shown in Figure 1 and Table 1. The GC

![Figure 4. FFA production of cells harboring each of the four mutations. The expression of mutated 'TesA was induced with 0.3 mM IPTG. The black bar indicates FFA production of E. coli MG1655 harboring WT 'TesA. These results indicate that the isolated 'TesA mutants directly increase FFA production compared to the WT 'TesA without any concerns regarding other mutations on the chromosomal DNA. With the exception of our earlier work, no studies have reported the overproduction of FFA caused by amino acid alterations in 'TesA. Therefore, the 'TesA mutants with increased functionality identified in the current study provide proof that combining the fluid array device and biotechnological approaches can accelerate the production of FFA-derived chemicals and biofuels.](Image)

**Table 1. Mutation Characterization for the Screened 'TesA Mutants and the Number of Overlapped Mutation Residues over the Total Screened Mutants**

| mutations on 'TesA | number of overlapped mutations (percentage) | fold increase in FFA production | remarks |
|--------------------|---------------------------------------------|---------------------------------|---------|
| L7Q                | 6/18 (33.3)                                 | 1.3                             |         |
| L51R               | 3/18 (16.6)                                 | 1.3                             |         |
| D74G               | 2/18 (11.1)                                 | 1.7 same mutation (D74) from Shin et al. |
| V144A              | 2/18 (11.1)                                 | 1.4                             |         |
| L4I                | 1/18 (5.5)                                  | 1.2                             |         |
| I6V                | 1/18 (5.5)                                  | 1.3                             |         |
| S47P, E143K        | 1/18 (5.5)                                  | 1.3                             |         |
| P24P, L51P, L104L  | 1/18 (5.5)                                  | 1.2                             |         |
| S10N, T84P, S122N, A171T | 1/18 (5.5) | 1.2 same mutation (A171) from Shin et al. |

CONCLUSIONS

Conventional HTS approaches for microbial screening suffer from difficulties in compartmentalization and throughput. Our previous research has attempted to overcome such limitations by applying two different screening pressures that were genetically designed. In this study, we utilized a hybrid screening approach composed of two screening strategies:
growth-based screening and reporter gene-based screening. A random library of $10^6$ mutants was prepared and screened, resulting in the identification of several TesA mutants that overproduce fatty acids, showing an almost two-fold increase compared to the WT TesA. Four mutants (TesA with I6V, L7Q, L51R, and D74G) accounted for approximately 70% of isolated mutants, indicating that the screening system used in this study successfully isolated FFA-overproducing cells from the large mutant library. Finally, we believe that the HTS application used in this study and the isolated TesA mutants may introduce a new direction for the microbial production of fatty acids throughout the random engineering of the target gene.

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### Author Contributions

J.W.L. and K.S.S. authors contributed equally to this work. J.W.L. and K.S.S. designed and conducted most of the experiments, analyzed the results, and wrote the manuscript. Y.S.R. conducted fundamental microbial experiments. Y.L. analyzed the location of the mutation in TesA. S.K.L. and T.K. designed the experiments, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported by the Next-Generation BioGreen 21 Program funded by the Ministry of Agriculture, Food and Rural Affairs (SSAC, PJ013457 and PJ013357).

## ABBREVIATIONS

ACP, acyl carrier protein; acyl-CoA, acyl-coenzyme A; FACS, fluorescence-activated cell sorting; FdR, multifunctional dual regulator of fatty acid metabolism in E. coli; FAS, fatty acid synthetic pathway; FFA, free fatty acid; HTS, high-throughput screening; GC, gas chromatography; IPTG, isopropyl-β-D-galactopyranoside; PDMS, poly-dimethyl siloxane; OD_{600}, optical density at wavelength of 600 nm; RFP, red fluorescent protein; TesA, leader sequence deleted thioesterase I from E. coli; TetA, tetracycline resistance protein; UV, ultraviolet light; WT, wild type

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