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Bacterial cell surface display of lipase and its randomly mutated library facilitates high-throughput screening of mutants showing higher specific activities

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This paper is dedicated to the retirement of Prof. Joon-Shick Rhee who has been pioneering in lipid biotechnology

Abstract

The thermostable lipase (TliA) from *Pseuodmonas fluorescens* was functionally displayed on the surface of *Escherichia coli* using the ice-nucleation protein (INP) as an anchor. The INP–TliA fusion proteins were correctly synthesized and localized on the surface, confirmed by flow cytometer and halo forming activity on tributyrin emulsion agar plate. Lipase-displaying cells were used as an alternative immobilized biocatalyst to hydrolyze olive oil in aqueous–organic solvent two phases reaction. Furthermore, the randomly generated library of TliA was also displayed on *E. coli*. In order to be able to screen mutants showing increased specific activities, we optimized culture conditions, induction condition and host cell types. From more than 10^5 members of library, top four mutants were selected. Selected clones of T48, T54, T61, and T68 showed 29-, 24-, 2-, and 19-fold increases, respectively, in whole-cell activities compared to wild-type enzyme. The DNA sequencing showed that one or three amino acids were exchanged and positions critical for increased activities were random. These results demonstrate that surface display provide a useful technology for directed evolution of industrially important lipases.

Keywords: Lipase; Directed evolution; Surface display; Ice-nucleation protein

1. Introduction

The surface display of lipases [triacylglycerol acylhydrolases, EC3.1.1.3] widely used for a variety of bio/chemical reactions including lipid hydrolysis, ester synthesis, and optical resolution, provides new source of immobilized biocatalysts [1–5]. Some microbial lipases such as *Humicola* lipase and *Fusarium* cutinase displayed on the yeast cell wall showed a low enzyme activity towards *p*-nitrophenyl butyrate, but not towards an emulsion of olive oil [3]. Washida et al. reported the functional display of *Rhizopus oryzae* lipase (ROL) on *Saccharomyces cerevisiae* via C-terminal half of α-agglutinin motif with long linker peptides [4]. More recently, a new yeast display system utilizing the flocculation functional domain of Flo1p was developed and used for the functional display of ROL by N-terminus fusion and the bio-diesel

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production in a solvent-free and water-containing system [5]. However, display of lipase on bacterial cells has not been reported yet.

The surface display technology also provides high-throughput screening (HTS) environment for directed evolution of enzymes, because it is easy to correlate one particular property of an enzyme to a selectable phenotype of a living cell [2]. Yeast surface display was used for the construction of ROL library with the combinatorial mutation of the lid domain, and the mutants with altered chain length specificity were screened by halo assay [6]. Previously, we have demonstrated the selection principle of cellulase mutants showing increased specific activities using bacterial surface display [7]. In this study, the extracellular lipase (thermostable lipase (TliA)) from *Pseudomonas fluorescens* SIK W1 was, for the first time, displayed on *Escherichia coli*, and followed by display of randomly mutated TliA library, useful in isolating selectively mutant enzymes showing improved specific activities. In doing so, the ice-nucleation protein (INP)-based bacterial display system was used [7–10]. To directly and completely screen more than 10^5 library, we optimized the visual screening procedure on agar plate with HTS instruments.

2. Materials and methods

2.1. Biological and chemical materials

For bacterial culture, Luria–Bertani (LB) medium containing yeast extract, 5 g/l; tryptone, 10 g/l; and NaCl, 5 g/l was used. When required, ampicillin was added to the final concentration of 100 μg/ml. For induction of protein synthesis, isopropyl-β-D-thiogalactoside (IPTG) was added up to 1 mM. The recombinant cells were washed with phosphate-buffered saline (PBS) solution and used for the characterization of lipase displayed on the cell surface. Most solvents and chemicals were purchased from Sigma–Aldrich, USA, as analytical grade.

2.2. Bacterial strains and plasmids

E. coli JM109 (*endA1 recA1 tyrA thi-1 hsdR17* (r_k^-, m_k^+) *relA1 supE44* Δ(*lac-proAB*)/F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15) and TG1 (*supE hsd 5thi* Δ(*lac-proAB*)/F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15) were used for DNA manipulation and display of lipase. The surface display vectors, pGF101 and pGF102, were constructed using pKK223-3 (Pharmacia, Uppsala, Sweden) as a backbone. An *inaK* gene encoding INP, a full length anchor motif, was obtained from pEIN229 [9] and subcloned into pKK223-3, resulting INP expression under the tac promoter control. Multicloning cites (*Xma*I, *Pst*I, *Sph*I, *Nhe*I, *Pvu*I, *Apa*I, *Hin*dIII) was inserted into the downstream of *inaK* gene, resulting pGF102 plasmid. The *inaK* gene in pGF102 was replaced by *inaKnc* gene from pANC3 [8] and named pGF101.

The DNA fragment encoding TliA was amplified by PCR using pTOTAL as a template [11,12]. The oligonucleotide sequences, 5′-AAGAGAGACCCCGGGA-TGGGTGTATTTGA-3′ and 5′-TACACCTCGCA-GGTAAAGCTTAAACGCAT-3′, were used as forward and reverse primers. The *Xma*I and *Hin*dIII sites are underlined. The amplified PCR products were digested with *Xma*I and *Hin*dIII and inserted the same restriction enzyme digested pGF101 and pGF102, resulting pJHC11 and pJHC12, respectively. For free form expression of TliA, *tliA* gene was also PCR-cloned into *Eco*RI and *Hin*dIII sites of pKK223-3, resulting pJHC18 plasmid.

2.3. Display of TliA on E. coli

Overnight culture of recombinant *E. coli* cells was transferred to new LB medium and grown to $OD_{600\,nm} = 0.4$. Synthesis of recombinant proteins was induced with 1 mM IPTG and incubated for an additional 6 h at 25 °C. For confirmation of surface localization of recombinant lipase, flow cytometric analysis was done. For immunofluorescence staining, 10^{10} cells were harvested and washed three times with PBS. The washed cells were resuspended in 1 ml PBS containing 1% skim milk and rabbit anti-TliA antibody (1:1000) [11] and incubated on ice for 1 h. After washing with PBS three times, the cells were incubated with FITC-conjugated anti-rabbit IgG antibody (1:100) on ice for 1 h. The FITC-labeled cells were examined under a FACScan flow cytometer (Becton Dickinson, Oxnard, CA).

2.4. Olive oil hydrolysis of TliA-displayed cells

Ten milliliters of recombinant *E. coli* cells which were prepared from 10 ml overnight cultures and washed with PBS was vigorously mixed with 10% olive oil in isooctane at 30 °C [13]. Released fatty acids were determined by cupric acetate method using oleic acid as a reference fatty acid.

2.5. Random mutagenesis and library display of TliA

A 1.4 kb *tliA* gene was amplified by error-prone PCR (EP-PCR) using pJHC12 as a template [14]. Oligonucleotide sequences for amplication were 5′-GATATTGTCGATAAACCCGACGAG-3′ as the upstream primer (YSK-5) and 5′-GATTTAATCTG-TATCAGGCTGAAA-3′ as the downstream primer (YSK-6). EP-PCR was performed under the following conditions: a total volume of 50 μl of 10 mM Tris–HCl pH 8.3; 40 mM KCl, 1.5 mM $MgCl_2$, 0.64 mM $MnSO_4$, 0.04 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, 2 μM each of primer YSK-5 and YSK-6, 5 ng of template DNA, and 2.5 U of *Taq* polymerase (Bioneer Inc., Daejeon, Korea). The PCR cycling condition was 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s. The amplified DNA fragments were digested with *Xma*I and *Hin*dIII, inserted into same enzyme digested pGF102, and transformed into *E. coli* cells by electroporation.

2.6. Screening procedure

Plasmid DNA was purified from *E. coli* JM109 harboring a library of TliA mutants and transformed into *E. coli* TG1 for visual screening. *E. coli* TG1 transformants were cultured overnight at 37 °C and transferred to new LB medium. After 12 h additional culture at 37 °C, the culture broth was serially diluted and about 10^5 cells were spread on LB medium agar plates containing 100 μg/ml ampicillin and 0.5% tributyrin (LBT) without IPTG. Plates were incubated at 25 °C for 24–72 h and consequently the colonies forming the halo more rapidly and largely were selected, and transferred onto new LBT agar plates. The selected clones were cultured in LB medium and whole-cell lipase activity was assayed.

2.7. Lipase activity assay

Lipase activity was assayed quantitatively by spectrophotometric method using *p*-nitrophenyl palmitate (pNPP). The pNPP was dissolved in acetonitrile at a concentration of 10 mM, and ethanol and 50 mM Tris–HCl (pH 8.5) buffer were subsequently added to a final ratio of 1:4:95 of acetonitrile:ethanol:buffer. 10^8 recombinant cells were added to 3 ml of pNPP solution and incubated at 45 °C for 10 min. The reaction was terminated by adding 2 μl of 0.5 M EDTA, and the lipase activity was detected by measuring the absorbance at 405 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min [15].

For a more precise assay of whole-cell lipase activity, 4-trifluoromethylumbelliferyl (coumarin) oleate from Marker Gene Technologies Inc., USA, was used as a fluorescence substrate. Recombinant cells (3×10^8) were mixed with 100 μM fluorescent substrates and the emission of fluorescence was measured at 510 nm with SpectraMAX GEMINIXS microplate fluorometer (Molecular Devices, USA).

3. Results and discussion

3.1. Display of TliA on E. coli

The extracelluar lipase, TliA, from *P. fluorescens* widely used for organic synthesis [10] was displayed on the surface of *E. coli* cells by using the ice-nucleation protein derived from *P. syringae*. The internal repeating domain-deleted INP (INPNC) as well as the INP whole protein were used as anchoring motifs. To investigate whether the full-size fusion proteins were properly synthesized, the whole-cell lysate were analyzed by SDS-PAGE and Western blot as described previously [8–10], resulting that the correct fusion proteins were detected (data not shown). The surface presence of TliA on *E. coli* cells was verified by a fluorescence-activated cell sorter (FACS) analysis (Fig. 1). The *E. coli* cells were probed with primary rabbit polyclonal antibodies reactive with TliA, and thereafter fluorescently stained using a FITC-labeled secondary antibody. The data demonstrate that the negative cells did not react with anti-TliA antibodies, and thus the JM109/pKK223-3

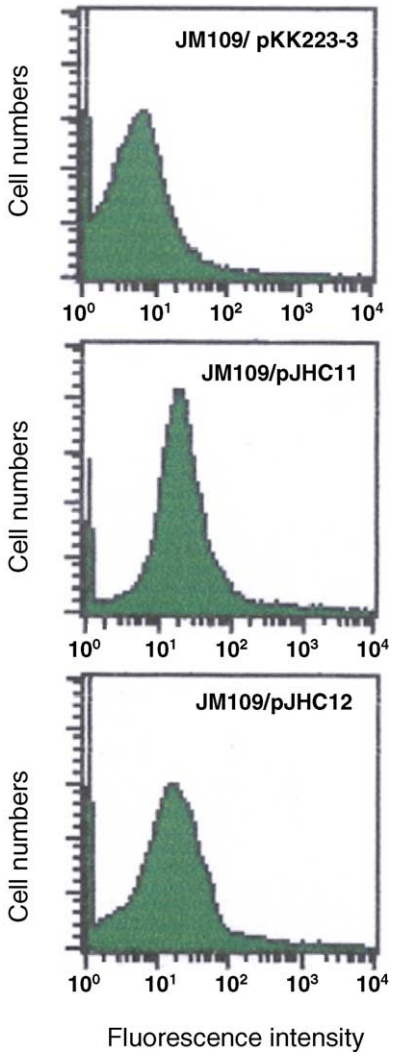


Fig. 1. Flow cytometry analysis of recombinant *E. coli* cells probed with rabbit antibodies reactive with TliA, and stained with FITC-labeled anti-rabbit IgG antibody. The histograms show that non-displaying cells (JM109/pKK223-3) gave a mean fluorescence of 7. The positive cells, JM109/pJHC11 and JM109/pJHC12, resulted in mean fluorescence values of 28 and 23, respectively. The number of cells analyzed in each experiments were 50,000.

cells showed to the left of the histograms. However, the probing of the positive cells, JM109/pJHC11 and JM109/pJHC12, with antibodies reactive with TliA resulted in increases of mean fluorescence values, indicating that the cells displayed numerous copies of TliA in an accessible form at the cell surface.

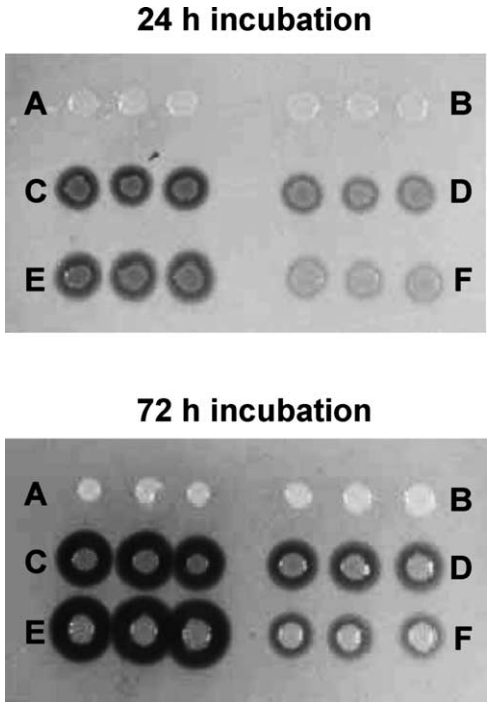


Fig. 2. Confirmation of functional display of TliA with halo-forming activities on tributyrin emulsion agar plate. The colonies are JM109 cells containing pKK223-3 (A), pEIN229 (B), pJHC11 (C), pJHC12 (D), pJHC18/pABC-ACYC (E), and pJHC18 (F), respectively.

In order to prove the functional display of TliA, whole-cell lipase activity was measured using *p*-nitrophenol palmitate as a chromogenic substrate. *E. coli* JM109/pJHC11 expressing the INPNC–TliA fusion proteins showed two times higher activity than JM109/pJHC12 expressing the INP–TliA fusion proteins did (data not shown), which is also qualitatively shown in Fig. 2. On the tributyrin emulsion agar plate, the cells containing the control vector (Fig. 2A) and expressing the INP (Fig. 2B) and TliA intracellularly (Fig. 2F) did not form the halos around their colonies during incubation for 24 h at 25 °C, while the cells displaying (Fig. 2C and D) or secreting TliA (Fig. 2E) formed the halos, suggesting that TliA were actively expressed and displayed on the surface of *E. coli* cells.

This functional display of lipase on bacterial cell surface is the first report, indicating that the INP-display system has a potential for the development of whole-cell biocatalysts.

3.2. Whole-cell hydrolysis of olive oil

The display of lipase on the cell surface is of immediate interest in the construction of novel biocatalysts, which has several advantages, e.g., in vivo immobilized biocatalysts to be easily reproduced and the ease of separating products from catalysts. We tried to use TliA-displayed *E. coli* cells as whole-cell biocatalysts for hydrolysis of olive oil in aqueous–organic solvent two-phase reaction system. In reaction, 10% (w/v) olive oil in isooctane was vigorously mixed with *E. coli* cells displaying TliA which was IPTG-induced at 25 °C, washed and resuspended in PBS (pH 7.2). As shown in Fig. 3, hydrolyzed fatty acids were released up to 45 μmole/ml with JM109/pJHC11 and 25 μmole/ml with JM109/pJHC12 after 30 h reaction, while only 10 μmole/ml fatty acids were detected with JM109/pJHC18 expressing TliA intracellularly. These results indicate that displayed TliA was functionally active to both artificial (*p*-nitrophenol palmitate) and real substrates (olive oil). As INP was reported to be actively expressed in other Gram-negative bacteria [16], these results would be easily applied for making more stable in vivo immobilized lipase biocatalysts in other bacteria.

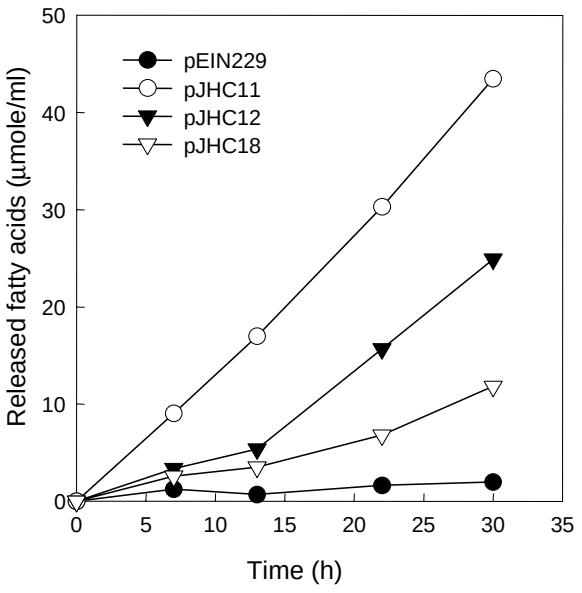


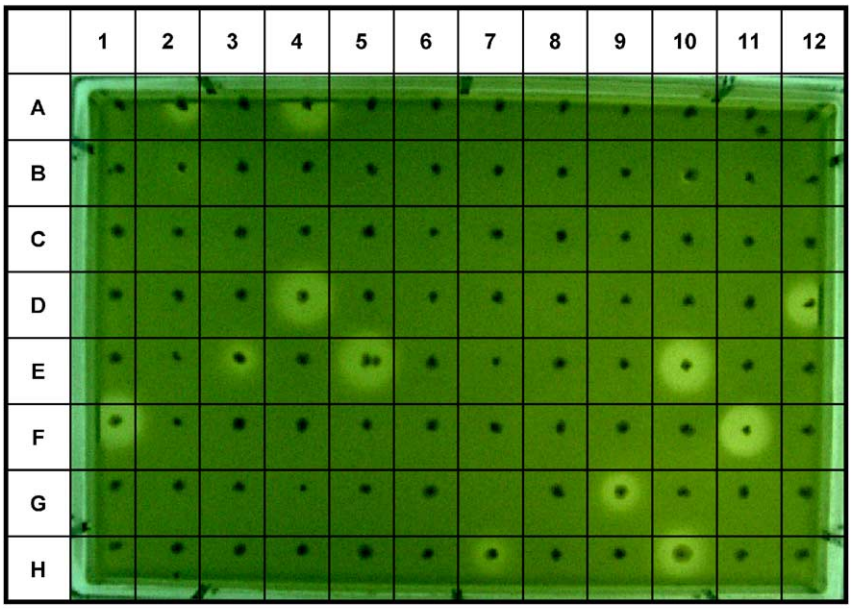
Fig. 3. Olive oil hydrolysis with surface-displayed TliA in aqueous–isooctane two-phase reaction system. The reaction was carried out with PBS-washed cells at 25 °C in vigorously shaking incubator at 150 rpm agitation speed.

3.3. Display and screening of TliA library

In order to screen out the mutant enzymes showing higher specific activities, a library containing more than 10^5 members of TliA mutants generated by error-prone PCR was displayed on *E. coli* JM109 cells using INP as an anchor. And then, for visual screening, the library clones were automatically picked on LBT agar plate containing 1 mM IPTG, 100 μg/ml ampicillin, and 0.5% tributyrin. From 15,000 clones incubated at 25 °C, the top 50 colonies showing the lipase halos were selected, compared to wild-type TliA-displaying ones. However, only 10 clones had higher whole-cell lipase activity measured with pNPP than wild-type clone, JM109/pJHC12, did. Upon DNA sequencing of top three mutants, one had no base pair substitution and two mutants has changed aspartic acid (D) to glutamic acid (E) at 200th amino acid, indicating that the above screening condition gave rise to select large number of false positives. There was no close correlation between the halo forming activity and whole-cell enzyme activity.

Thus, we tried to optimize the visual screening protocol including culture conditions, induction method and host cell types. At first, 16 *E. coli* strains available at laboratory were tested for the uniformity of colonies and halos on agar plate, background lipase activities of the cells, and even display of lipase. Among them, *E. coli* TG1 was selected as a library screening host cell. When the TliA library was displayed on TG1, the negative clones showing lower or no lipase activities were easily selected out. Furthermore, for cultivation, 500 μl multi-well plate cultures incubated overnight at 37 °C with vigorous shaking were performed at first. For uniform induction, the clones from multi-well plates cultures were automatically picked with the QPix robotic colony picker on LBT agar plate containing 100 μg/ml ampicillin and 0.5% tributyrin without IPTG, followed by incubation at 25 °C over 2–3 days for screening. Good correlation was obtained between the halo forming activities (Fig. 4A) and whole-cell lipase activities (Fig. 4B). Our previous report on cellulase evolution demonstrated that mutant enzymes showing increased specific activities were selectively screened from

(A)



(B)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.6	9.1	0.5	22.1	0.6	0.8	0.8	0.6	0.7	0.7	0.6	0.6
B	0.8	1.0	0.9	0.6	0.7	0.6	0.7	1.0	0.6	0.6	0.7	0.7
C	0.8	0.7	0.7	0.8	0.7	0.8	0.6	0.7	0.9	0.6	0.8	0.7
D	0.6	0.7	0.8	31.9	0.6	0.7	0.6	0.7	0.6	0.5	0.8	27.0
E	0.7	0.7	13.2	1.0	29.3	0.8	0.8	0.7	0.8	34.5	0.6	0.6
F	24.3	0.6	0.7	0.6	0.7	0.8	1.5	0.7	0.6	0.7	98.9	0.5
G	0.8	0.6	0.8	0.6	0.9	0.6	0.0	0.7	0.7	0.8	0.7	0.6
H	0.6	0.7	0.5	0.6	0.8	0.7	10.5	0.8	0.7	24.8	0.8	0.6

Fig. 4. Correlation between the halo forming activities on agar plates (A) and whole-cell lipase activities (B). The colonies were automatically picked from 500 μl multi-well plate cultures to tributyrin emulsion agar plates followed by incubation at 25 °C for optimal induction. Whole-cell lipase activities were measured with chromogenic substrate, pNPP, and are shown in absorbance of reaction mixture at 405 nm per optical density of the cells at 600 nm.

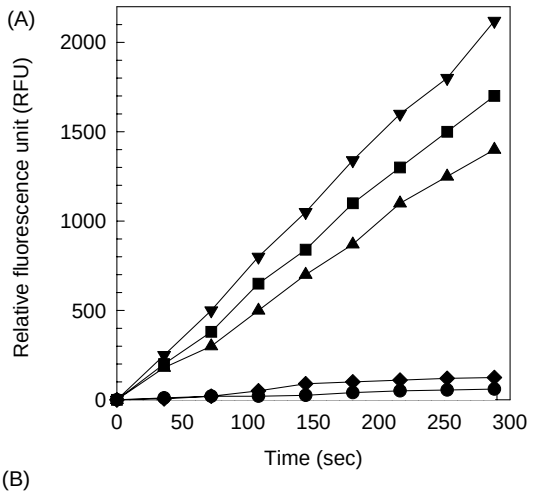
surface displayed enzyme library [7]. That was based on uniform expression mediated by the INP anchor. Here, top 115 clones were selected from 10^5 library and their whole-cell lipase activities were measured with pNPP. Upon comparing activities of mutants with wild-type enzyme, 50% mutants showed about 20% increase in whole-cell activities. One-fourth of mutants showed more than 20% increase, including one mutant showing 16 times higher whole-cell activity. The other one-fourth of mutants showed up to 25% decrease of activities. Finally, we selected top four clones such as T48, T54, T61, and T68.

3.4. Characteristics of selected mutants

The clones T48, T54, T61, T68 and wild-type in TG1 were independently cultured in test tube and induced with 1 mM IPTG, and then washed with PBS. We compared more precisely whole-cell activities of selected mutants based on 3×10^8 cells/ml with fluorescent substrate, coumarin oleate, as shown in Fig. 5A. Each clones of T48, T54, T61, and T68 showed 29-, 24-, 2-, and 19-fold increases in activities, respectively.

Fig. 5B shows the base pair substitutions and corresponding amino acid changes. Although one or three amino acids were identified, positions critical for increased activities were not clear, which needs further rounds of evolution. 1–3 silent mutations were also involved in every mutant. All the mutations found in four selected mutants were randomly distributed, but none were found in the C-terminal glycine rich box (374–476 amino acids) responsible for secretion through ABC (ATP-Binding Cassette) transporter. Surprisingly, there were no amino acid changes near the active site (GXSXG, 205–209 amino acids) and oxyanion hole (136–142 amino acids), which are contacted to substrates and play a major role in catalysis. Currently, 3D structure is not available for TliA and related subfamily I-3 of lipolytic enzymes [17]. Even though directed evolution of TliA could provide a logical way to identify sensitive positions for increasing activity without knowing 3D structure, solved structure would greatly facilitate the interpretation of mutations at the specific amino acid residues in improved lipase mutants.

(A)



(B)

Mutant	Base substitutions	Amino acid changes
#T48	A23G, A40G T169C, T214A A1098G, T1248C	N8S,S14G W72R
#T54	A1081T, A1098G	N361Y
#T68	C732T, T805C, C968T, A1067G, A1098G	S269P T323M, Q356R
#T61	T71C, A1098G	I24T

Fig. 5. Lipase activities (A) and DNA base substitution and corresponding amino acid exchanges of selected mutants (B). The lipase activities were measured with fluorescence substrate, coumarin oleate. The symbols indicates wild-type (●) and mutants of T48 (▼), T54 (■), T61 (◆) and T68 (▲), respectively.

4. Conclusion

E. coli cell surface-displayed lipase was obtained by in vivo immobilization with the aid of the INP-display system. In aqueous–organic solvent two-phase reaction, olive oil was well hydrolyzed with surface-immobilized lipases. The INP-display system also provided a good HTS environment for directed evolution of bacterial lipase enzymes.

Acknowledgements

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