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High-throughput screening methods for selecting L-threonine aldolases with improved activity

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Abstract

More and more, aldolases are being recognized as useful catalysts that carry out the reversible addition of a ketone donor to an aldehyde acceptor in achieving high stereoselectivity. Threonine aldolases catalyze the synthesis of variable β-hydroxy-α-amino acids, which are important structural units of various antibiotics and immunosuppressants. However, the enzymatic properties need to be improved to support a broader application to synthetic chemistry. Although directed-evolution is a powerful tool for improving enzymatic properties, the successful outcome depends on the efficiency of screening systems. We designed and proposed two high-throughput screening schemes for selecting L-threonine aldolase mutants with improved properties. These schemes utilized the toxicity of aldehyde, which acts as an acceptor in the aldol condensation. In these schemes, the following occurs: (1) the higher L-threonine aldolase activity reduces the toxic effect of aldehyde, which leads to the survival of the corresponding clone (the positive-selection scheme), and (2) the higher L-threonine aldolase activity produces more toxic aldehyde, which causes the death of the corresponding clone (the negative-selection scheme). According to the positive-selection scheme, we successfully selected L-threonine aldolase mutants with higher activities than the wild-type, from a randomly generated LTA library.

Keywords: Aldehyde; L-Threonine aldolase; Directed-evolution; High-throughput screening; *Pseudomonas aeruginosa*

1. Introduction

Aldolases are a group of enzymes responsible for the reversible-stereoselective C–C bond formation. Aldolases have attracted many researchers' attention because they can catalyze the asymmetric C–C bond formation [1–5], which is one of the most-challenging themes in synthetic organic chemistry. Thus, the use of aldolase is an interesting alternative to the chemical aldol reaction because of the mild reaction conditions, the high stereoselectivity of the enzymatic reaction, and the minimal use of protective-group chemistry [5–7].

Threonine aldolase is a pyridoxal 5-phosphate-dependent enzyme [8], which catalyzes the cleavage of threonine to glycine and acetaldehyde, as well as the reverse reaction, aldol condensation (Scheme 1). Threonine aldolase stereospecifically catalyzes this interconversion and accepts a broad range of substrates. Thus, this enzyme has been used extensively for the resolution or synthesis of β-hydroxy-α-amino acids, which are important structural units of various

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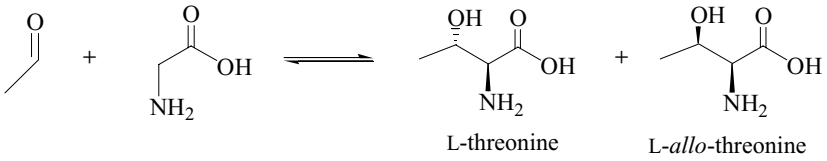

Scheme 1. LTA-catalyzed reaction.

antibiotics and immunosuppressants [9–16]. In addition, threonine aldolase takes non-phosphorylated compounds as substrates, unlike other aldolases that need phosphorylated substrates. The preparation of phosphorylated compounds is usually laborious, and most of the synthetically useful products are not phosphorylated. Therefore, threonine aldolase is regarded as a potentially useful catalyst in synthetic chemistry. However, the use of enzymes in synthetic chemistry has been limited due to their low activity towards non-natural substrates and their low stability in organic solvents. Furthermore, enzymes have not evolved to gain the highest activity even towards natural substrates. For broader applications of threonine aldolase in synthetic chemistry, we need to improve the enzyme properties, mainly the enzyme activity, the altered substrate specificity, and the high stability in organic solvents. Recently, the directed-evolution approach has proved to be a powerful tool for modifying natural enzymes regardless of structural information or the detailed information about their catalytic mechanism [17–20]. Directed-evolution is an iterative process of random mutagenesis and screening/selection to accumulate beneficial mutations until a desired property is attained [21–23]. The successful outcome of directed-evolution is largely dependent on the efficiency of screening methods. A coupled-enzyme assay has been used as a screening method for KDPG aldolase mutants with modified activities through directed-evolution [24,25]. However, the coupled-enzyme assay requires expensive NADH, and it is laborious because each clone should be tested separately. Furthermore, NADH could be consumed by contaminated enzymes when unpurified aldolases such as cell extracts are used. If this were to happen, the specified activities of screening clones could be misjudged.

In this study, we observed that high concentrations of aldehyde, which acts as an acceptor in the aldol condensation reaction, are toxic to *Escherichia coli*. Based on this observation, we designed and proposed high-throughput selection schemes for screening aldolase mutants with modified activities. For this purpose, we used L-threonine aldolase (LTA) from *Pseudomonas aeruginosa*. LTA can remove and generate acetaldehyde by aldol condensation and aldol cleavage, respectively. Therefore, it is possible that higher LTA activities alleviate the toxic effect of acetaldehyde on *E. coli* cell growth in the presence of high concentrations of acetaldehyde. This phenomenon can be used for designing a positive-selection scheme to screen mutant clones with higher LTA activities. On the other hand, clones with higher LTA activities can generate more acetaldehyde in the presence of L-threonine, and thereby increase the toxic effect of acetaldehyde. Likewise, this phenomenon can be used for designing a negative-selection scheme to screen clones that show growth defects in the presence of L-threonine due to their higher LTA activities. Here, we show that the two selection methods are possible. In addition, and according to the positive-selection scheme, we have successfully selected clones with higher LTA activities from a randomly generated LTA library.

2. Experimental

2.1. Plasmids and bacterial strains

Plasmid, pSS6-1 was used as a vector for cloning, sequencing, and the library construction [26]. Plasmid, pET-22b, purchased from Novagen, was used for expression. For the construction and propagation of plasmids, the bacterial host was *E. coli* JM109 [28]. For protein-purification using the pET expression vector system, we used *E. coli* BL21(DE3) [29]. *E. coli* TOP10, purchased from Invitrogen, was used as the host for the construction of random library.

2.2. Materials, enzymes, and chemicals

Genomic DNA of *P. aeruginosa* (ATCC 47085) was purchased from American Type Culture Collection. *Pwo* DNA polymerase was purchased from Roche. *Taq* polymerase, T4 DNA ligase, and all restriction enzymes were purchased from Promega. Ni-NTA agarose resin for His-tagged protein purification was purchased from QIAGEN. The yeast alcohol dehydrogenase and all other chemical reagents were purchased from Sigma.

2.3. Plasmid construction

A 1038 bp LTA-coding sequence [27] was amplified from genomic DNA of *P. aeruginosa* by a standard polymerase chain reaction (PCR) with primers LTA-F (5′-TAGAATTCATGACCGATCACACCCAACAG-TTC-3′) and LTA-R (5′-TAGGATCCTCAGGCGCC-CATCACCAGGCG-3′) flanking the gene with restriction sites *Eco*RI and *Bam*HI, respectively (underlined). PCR amplification was performed using *Pwo* polymerase, a high fidelity proofreading polymerase. The amplified fragment was purified from agarose gel, digested with *Eco*RI and *Bam*HI and inserted into the *Eco*RI-*Bam*HI site of the pSS6-1 vector, yielding the plasmid pSS6-LTA. The LTA-coding sequence in pSS6-LTA was used as a template in error-prone PCR to generate random mutations.

Random mutagenesis was carried out using error-prone PCR with primers LTA-F and LTA-R [24]. The reaction conditions were: 20 ng template/10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.5 μl DMSO/0.2 mM $MnCl_2$/0.2 mM dNTP/5 U *Taq* polymerase/40 pmol of each of the primers, in a total volume of 100 μl. The mixture was thermocycled for 30 rounds at 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min, and then one round at 72 °C for 7 min. The mutated fragments were purified from agarose gel and then digested with *Eco*RI and *Bam*HI. The resulting fragment was cloned into pSS6-1 and transformed into *E. coli* TOP10.

For protein purification, the LTA-coding sequence in vector pSS6-1 was moved to pET22b. For this purpose, the coding sequence was amplified by PCR, using primers pETLTA-F (5′-CACAGGAAACAGAA-CATATGACCGATCACAC-3′) and pETLTA-R (5′-CTGCAGGTCGACGGACTCGAGGGCGCCCATC-ACCAG-3′) flanking the gene with restriction sites *Nde*I and *Xho*I, respectively (underlined). The amplified fragments were purified from agarose gel and then digested with *Nde*I and *Xho*I. The resulting fragments were cloned into the *Nde*I-*Xho*I sites of pET-22b.

2.4. Determination of relative colony appearance

We examined the effects of the overexpression of LTA on bacterial growth in the presence of L-threonine. To accomplish this, we did the following: (1) we transformed JM109 cells with plasmid pSS6-LTA and (2) we plated them on M9 minimal agar plates containing 0.2% glucose, 0.2% L-threonine, 50 μg/ml of ampicillin, and variable concentrations of IPTG. Then, we incubated the plates at 37 °C for 30 h. We calculated the relative colony appearance (RCA) as a ratio of the number of colonies appearing on a selective plate to the number of colonies on the control M9 minimal agar plate containing 0.2% glucose and ampicillin (50 μg/ml). We assessed the toxicity of acetaldehyde by plating JM109 cells transformed with pSS6-LTA on LB agar plates containing 50 μg/ml of ampicillin along with variable concentrations of acetaldehyde. The RCA was calculated using the same LB plate without acetaldehyde as a control plate. We also assessed the removal of the toxic acetaldehyde by the overexpressed LTA in the following way: we plated JM109 cells transformed with pSS6-LTA on LB agar plates containing ampicillin (50 μg/ml), 16 mM acetaldehyde, along with variable concentrations of IPTG. The RCA was calculated using the same LB agar plate without acetaldehyde as a control plate.

2.5. Selection of improved LTA activities

We screened the mutant library by plating JM109 cells transformed with the plasmid library on LB agar plates containing ampicillin (50 μg/ml), 20 mM acetaldehyde, and 0.5 mM IPTG. Plasmid DNA from the survived clones was prepared and reintroduced into JM109 cells. We rescreened the resulting transformed cells on the same selective agar plates to confirm that the selected clones carried improved LTA activities.

2.6. Purification of recombinant LTA proteins

BL21(DE3) cells were transformed with the pET expression plasmids, which carried the wild-type or mutant LTA-coding sequence. These pET expression plasmids were used to express the hexahistidine-tagged (His-tagged) LTA proteins. The cells were grown in 250 ml of LB broth and induced by adding 0.5 mM IPTG when the absorbance at 600 nm reached ∼0.6. The cells were further grown for 2 h before being harvested. We resuspended the cell pellet with a lysis buffer [50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 10 μM pyridoxal 5-phosphate (PLP)], and then it was disrupted by sonication. After sonication, the supernatant fraction was subjected to Ni^{2+}-chelating affinity chromatography containing Ni-NTA agarose (Qiagen) equilibrated with the lysis buffer. Unbound proteins were washed off the column with the lysis buffer and then with a binding buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 10 μM PLP). Bound proteins were eluted with an elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole, 10 mM β-mercaptoethanol, 10 μM PLP). The recombinant protein-containing fractions were dialyzed against a storage buffer (50 mM NaH_2PO_4, pH 8.0, 1 mM DTT and 10 μM PLP), followed by concentration with a Centricon YM-30 (Amicon). The protein concentration of each purified enzyme was measured by the Bradford method using a Bio-Rad Protein Assay Kit [30].

2.7. Aldolase assay for aldol condensation

The aldol condensation activity was determined by the rate of depletion of acetaldehyde. The acetaldehyde concentration was determined by a method similar to that reported by Fong et al. [24]. The assay mixture contained 100 mM HEPES buffer, pH 8.0, 10 mM glycine, 10 mM acetaldehyde, 50 μM PLP, 1 mM DTT, and 5 μg of enzyme, in a final volume of 1 ml. The reaction mixture was preincubated at 30 °C for 10 min. Then, the reaction was initiated by adding LTA into the assay mixture. Aliquots of 25 μl were withdrawn from the reaction mixture at different time points and quenched with 30 μl of 7% perchloric acid. The samples were neutralized with 20 μl of 1 M NaOH and then diluted to 1 ml with 100 mM HEPES buffer, pH 8.0, containing 300 μM NADH and 30 U yeast alcohol dehydrogenase. The acetaldehyde concentration was calculated from the disappearance of NADH measured by the absorbance decrease at 340 nm.

2.8. Aldolase assay for L-threonine cleavage

Threonine aldolase activity was measured spectrophotometrically at 340 nm by a coupled-enzyme assay with alcohol dehydrogenase [31]. The assay mixture contained 100 mM HEPES buffer, pH 8.0, 300 μM NADH, 50 μM PLP, 30 U yeast alcohol dehydrogenase, 1 mM DTT, and appropriate amounts of the enzyme and L-threonine, in a final volume of 1 ml. We preincubated the mixture at 30 °C for 10 min. Then, the reaction was initiated by adding LTA into the assay mixture and the disappearance of NADH was monitored for 10 min by the absorbance decrease at 340 nm. Kinetic parameters were determined from double reciprocal plots of the initial velocity and the substrate concentration. One unit (1 U) of the aldolase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of acetaldehyde per minute at 30 °C.

3. Results and discussion

3.1. Overexpression of LTA in E. coli

The L-threonine aldolase gene was amplified from *P. aeruginosa* genomic DNA by PCR. The gene contains an open-reading frame consisting of 1038 nucleotides corresponding to 346 amino-acid residues. It displays a 94% sequence similarity with a low specificity LTA from *Pseudomonas* sp. NCIMB10558 [31]. The amplified fragment was cloned into a pSS6-1 vector, yielding pSS6-LTA (Fig. 1A). This construct contained the *tac* promoter and the LacI repressor, which control the protein-expression level. When LTA expression was induced by 0.5 mM IPTG in an *E. coli* strain JM109 containing pSS6-LTA, most of the induced protein was soluble (Fig. 1B). The induced protein migrated as a 36 kDa protein on a SDS-polyacrylamide gel, which matches the 38 kDa calculated from the deduced amino-acid sequence of LTA.

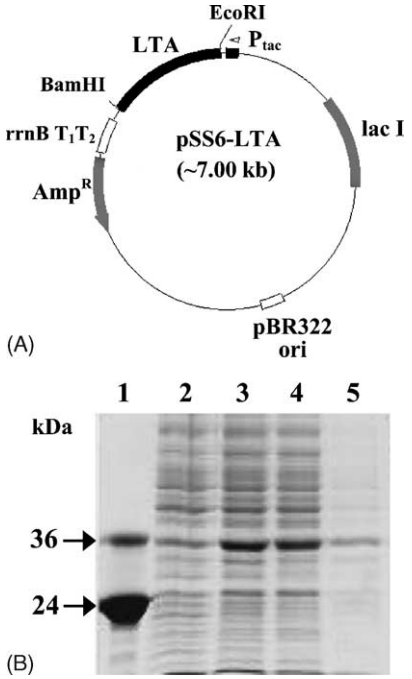

Fig. 1. Overexpression of the recombinant LTA in *E. coli*. (A) The map of plasmid pSS6-LTA. The LTA expression is under the control of the *tac* promoter and the *lac* repressor. (B) SDS-PAGE analysis of the LTA expression. *E. coli* JM109 cells containing pSS6-LTA were grown and induced with 0.5 mM IPTG. The cells were sonicated and then centrifuged to divide into two fractions, soluble and insoluble fractions. The proteins were fractionated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Lane 1, size markers; Lane 2, total proteins of the uninduced cells; Lane 3, total proteins of the IPTG-induced cells; Lane 4, soluble fractions of the IPTG-induced cells; Lane 5, insoluble fractions of the IPTG-induced cells.

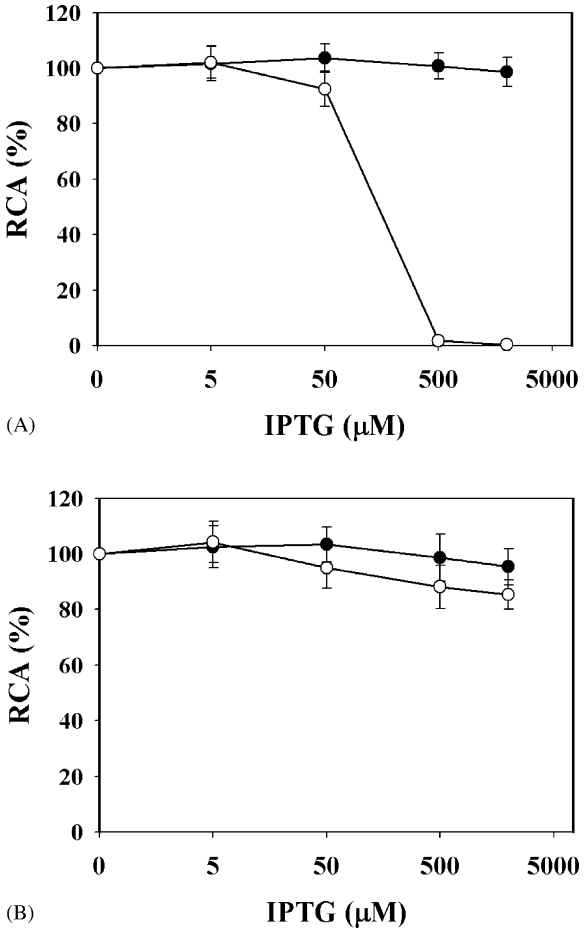

Fig. 2. The toxic effect of LTA overexpression on *E. coli* cell growth. (A) *E. coli* JM109 cells transformed with pSS6-LTA or the control pSS6-1 plasmid were plated on M9/ampicillin/0.2% L-threonine/0.2% glucose agar plates containing variable concentrations of IPTG. They were incubated at 37 °C for 30 h, and the relative colony appearance (RCA) was calculated as a ratio of the number of cells being plated on the selective plates to that on the same plate without IPTG. Symbols: (○) pSS6-LTA; (●) pSS6-1. (B) *E. coli* JM109 cells transformed with pSS6-LTA or pSS6-1 plasmid were plated on M9/ampicillin/0.5 mM IPTG/0.2% glucose agar plates without L-threonine. The RCA was calculated as a ratio of the number of cells being plated on the selective plates to the number of cells on the same plate without IPTG. Symbols: (○) pSS6-LTA; (●) pSS6-1.

3.2. Selection scheme

3.2.1. Effects of overexpression of LTA on E. coli cell growth

To test the effect of LTA activity on *E. coli* in the presence of L-threonine, *E. coli* JM109 was transformed with pSS6-LTA and then plated on M9/ampicillin/0.5 mM IPTG/0.2% L-threonine/0.2% glucose agar plates (Fig. 2A). We found that the relative colony appearance (RCA) decreased as the concentration of IPTG increased. However, the RCA of JM109 cells that were transformed with the control pSS6-1 plasmid remained unchanged. We also examined the RCA of JM109 cells transformed with pSS6-LTA on the same M9 plates without L-threonine. The addition of IPTG did not affect the RCA of JM109 cells transformed with pSS6-LTA (Fig. 2B), although the size of the colonies decreased slightly (data not shown).

These results suggest that the product(s) generated from L-threonine by the overexpressed LTA is toxic to *E. coli*. It is likely that the toxic product is acetaldehyde since the other LTA product is glycine, an amino acid.

3.2.2. Proposed scheme for negative selection

The generation of the toxic product from L-threonine, by the overexpressed LTA, can be used to screen LTA mutant derivatives with improved properties from a randomly generated LTA library. For example, the growth of cells overexpressing LTA mutants with higher activities is affected by <0.5 mM IPTG so that those clones will disappear on M9 media containing 0.2% L-threonine from the mutant library. Therefore, the negative selection is possible by picking the corresponding clones on the replica plates without IPTG or L-threonine.

3.2.3. Effects of high concentrations of acetaldehyde on E. coli cell growth

To confirm that high concentrations of acetaldehyde are toxic to *E. coli*, we examined the RCA of JM109/pSS6-1 or JM109/pSS6-LTA cells on LB/ampicillin agar plates containing acetaldehyde. As the concentration of acetaldehyde increased, the RCA decreased (Fig. 3), indicating that high concentrations ($\geq$16 mM) of acetaldehyde are toxic to *E. coli*.

3.2.4. Proposed scheme for positive selection

Since LTA can convert acetaldehyde to L-threonine with glycine, overexpression of LTA could lower the concentration of acetaldehyde. Thus, we tested whether overexpression of LTA increased the relative colony appearance (RCA) or not. JM109/pSS6-LTA cells were plated on LB/16 mM acetaldehyde agar plates containing variable concentrations of IPTG where JM109/pSS6-1 control cells did not grow (Fig. 4). We found that the RCA increased as the concentration of IPTG increased, indicating that overexpression of LTA reduces the toxic effect of acetaldehyde. About 90% of the cells survived in the presence of 0.5 mM IPTG. This result can offer a positive screening scheme for LTA mutants having higher activities. This screening scheme includes the selection of survival colonies on selection agar plates containing 20 mM acetaldehyde from an LTA mutant library, because no cells containing pSS6-LTA survived in the presence of 20 mM acetaldehyde and 0.5 mM IPTG (Fig. 3). This positive-selection scheme is very efficient because the whole library can be tested in a couple of days.

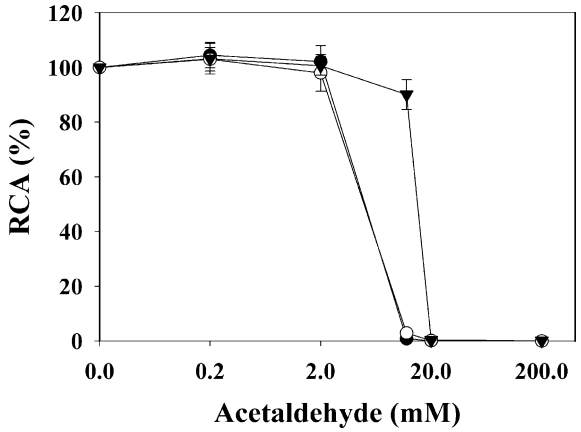

Fig. 3. The effect of acetaldehyde on *E. coli* cell growth. JM109 cells were transformed with pSS6-LTA or pSS6-1 on LB agar plates containing 50 μg/ml of ampicillin and variable concentrations of acetaldehyde with or without 0.5 mM IPTG. The relative colony appearance (RCA) was calculated using the same LB plate without acetaldehyde as a control plate. Symbols: (○) pSS6-LTA without IPTG; (●) pSS6-1 without IPTG; (▼) pSS6-LTA with IPTG.

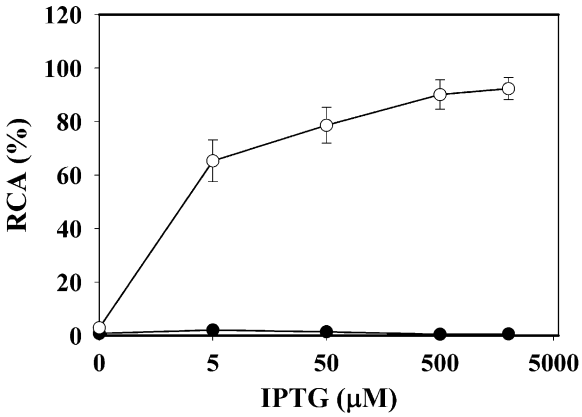

Fig. 4. Resistance to acetaldehyde in LTA-overexpressed cells. *E. coli* JM109 cells containing pSS6-LTA or pSS6-1 were plated on LB containing 16 mM acetaldehyde and variable concentrations of IPTG. The relative colony appearance (RCA) was calculated using the same LB plate without acetaldehyde as a control plate. Symbols: (○) pSS6-LTA; (●) pSS6-1.

3.3. Positive selection from a random LTA-mutant library

We constructed an LTA-mutant library by cloning the randomly mutagenized LTA-coding sequence with error-prone PCR into pSS6-1. Sequencing 10 clones, which were randomly chosen from the library, revealed an error rate of 0.33% (3–4 bp of the 1038-bp LTA coding sequence). This condition causes two or three amino-acid changes within each LTA mutant. We tested approximately 20,000 clones for improved LTA activities by the positive selection. Cells containing the randomly generated LTA mutant library were plated on LB/20 mM acetaldehyde/ampicillin/0.5 mM IPTG agar plates. Ten clones appeared on the selective plates, and then they were further tested. Plasmid DNA was prepared from each clone and used for retransformation into JM109. When the transformed cells were plated on the same selective plates, five clones showed colony appearance, but the wild-type did not. Sequence analysis confirmed that the five clones had two or three amino acid changes within each LTA sequence (data not shown). The mutants LTA proteins were purified in His-tagged forms and analyzed for their ability to catalyze the aldol condensation reaction with glycine and acetaldehyde as substrates (Fig. 5). Among the five mutant enzymes, LTA-S2 showed the highest specific activity. Compared with the wild-type, LTA-S2 exhibited a 2.1-fold increase in catalytic activity. We also determined kinetic parameters of the mutant enzymes for L-threonine cleavage using a standard coupled-enzyme assay (Table 1). Most of the mutant enzymes had increased V_{max} values for L-threonine.

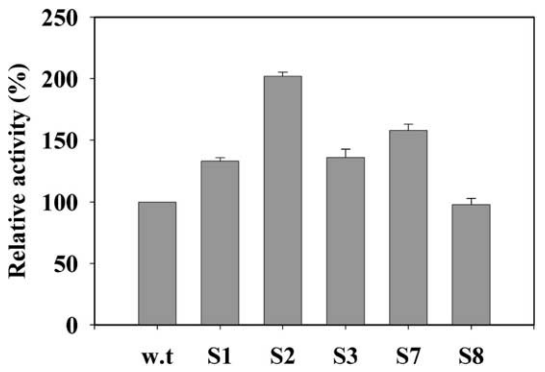

Fig. 5. Relative specific activity of LTA mutants for aldol condensation. Assays for the aldol condensation reaction were carried out with 10 mM acetaldehyde and 10 mM glycine. The relative activity represents the specific activity of LTA mutants relative to that of the wild-type LTA. Data shown are the average of three independent determinations.

Table 1
Steady-state kinetic parameters of LTA mutants for L-threonine cleavage

LTA	V_{max} (U mg^{-1})	K_m (mM)	V_{max}/K_m (U mg^{-1} mM^{-1})
w.t	30.31 (±1.72)	5.55 (±0.34)	5.47 (±0.16)
S1	29.21 (±0.97)	6.12 (±1.00)	4.77 (±0.58)
S2	38.21 (±0.90)	5.38 (±0.89)	7.10 (±0.80)
S3	42.10 (±1.05)	6.33 (±0.33)	6.65 (±0.27)
S7	35.62 (±0.05)	6.69 (±0.14)	5.32 (±0.06)
S8	40.18 (±2.26)	15.47 (±2.84)	2.66 (±0.35)

The steady-state kinetic parameters of the wild-type and mutant LTA enzymes for L-threonine cleavage were measured by means of a coupled enzyme assay as described in Section 2.8. Kinetic parameters were determined from Lineweaver-Burk plots. The values of the kinetic parameters are given as the means (±S.D.) of three different determinations.

We tested whether the five clones could be screened through the negative selection or not. All the clones did not produce enough acetaldehyde for its toxic effect on cell growth. This suggests that the positive selection may be the more sensitive screening method than the negative selection. However, it is noteworthy to mention that our selection methods could generate "false-positives" because the total cellular enzyme activity, not the specific enzyme activity, would be related with the cell growth at high concentrations of acetaldehyde [32]. For example, clones producing more enzymes without the elevated activity may be selected although these clones can be excluded by comparing their expression to that of wild type.

4. Conclusions

For the development of industrially useful biocatalysts, several properties of natural enzymes need to be improved. For example, the activity of natural enzymes towards both natural and non-natural substrates, and the stability of natural enzymes at high temperatures and in organic solvents should be increased. Over the years, directed-evolution proved to be a powerful approach for improving the properties of enzymes. Although L-threonine aldolase is also

a potentially useful biocatalyst, there is no efficient screening system. This lack of a screening system slowed-down the directed-evolution approach toward improving this enzyme.

This paper provides two potential selection methods based on the toxicity of aldehydes to bacterial cell growth. These methods can be used for screening L-threonine aldolase derivatives with modified properties. We successfully used the positive-selection methods to screen the L-threonine aldolase mutants that carry higher catalytic activities than the wild-type. Although we did not practically test, the negative-selection scheme we designed and proposed could be used as an alternative or complementary tool for screening L-threonine aldolase. These two selection methods may also be applied to other aldolases, which utilize toxic aldehydes as substrates, for a directed-evolution approach.

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