Selection of an Active Enzyme by Phage Display on the Basis of the Enzyme's Catalytic Activity in vivo

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We have developed a novel phage display method based on catalytic activity for the in vivo selection of an enzyme. To confirm the validity of our method and to demonstrate its potential utility, we used biotin protein ligase (BPL) from Escherichia coli *as a model enzyme. We were able to demonstrate the potential value of our method by selective enrichment for the* birA *gene, which encodes BPL, in a mixed library. The presented method for in vivo selection should allow selection of various enzymes that catalyze modification of peptides or proteins, such as protein ligase, acetylase, kinase, phosphatase, ubiquitinase, and protease (including caspase). The method should be useful in efforts to analyze mechanisms of signal transduction, to find unidentified enzymes encoded by cDNA libraries, and to exploit artificial enzymes.*

Introduction

The increasing demand for enzymes for medical, pharmaceutical, chemical, biological, and industrial applications has generated considerable interest in the engineering of enzymes with novel properties. Moreover, there is a demand for methods that allow for the in vivo selection of enzymes on the basis of their intracellular activities, which can be used for the elucidation of protein–protein interactions and the characterization of as yet unknown intracellular networks of enzymes. Development of artificial enzymes, the identification of novel enzymes, the analysis of unknown enzymes, and the in vivo characterization of intracellular networks of enzymes are also attractive goals from the perspective of molecular evolution.

Several basic approaches exist for the engineering of proteins. Specific mutations can be introduced at predetermined sites within a protein.[1–3] Alternatively, introduction of random mutations can be followed by in vivo or in vitro selection, and many variants can be assayed cheaply and rapidly.[4–9] The main advantage of in vivo methods of selection is that the properties of the selected proteins reflect the cellular environment. However, the diversity of sequence libraries is limited by the efficiency of transformation and by the nature of the protein in question, in particular, for housekeeping proteins and/or when over-expression is associated with intracellular toxicity.[5] The main advantage of selection in vitro is that the diversity of protein libraries is larger than can be achieved by in vivo selection, although the activity of the selected protein in the cellular environment is not necessarily guaranteed.[4,6–9]

Phage display is a powerful method for in vitro selection of functional proteins, including enzymes, from a random or combinatorial library with more than 10^8 different members.[4,6] The selection of proteins by their display on the surface of phage molecules, which is based on binding activity, has been well documented; however, the selection of enzymes on the basis of catalytic activity is significantly more difficult[10] since, for example, reaction products diffuse from the reaction site (Figure 1) and the properties of the displayed enzyme remain the same before and after the catalytic event. One way to solve this problem is to select the enzyme, which is displayed on a phage, by binding to a suicide inhibitor[11–13] or to a transition-state analogue that is connected covalently to a solid support (see Figure 2A).[14–18] However, binding to such an analogue does not necessarily guarantee a perfect correlation with the catalytic activity of the enzyme.[15,17]

Other methods have also been developed[19–21] that correlate the activity of a displayed enzyme with the specific binding of the product to a solid support by linking the product to the enzyme either directly or indirectly (Figure 2B). The enzyme is linked to its product by a strategy that makes use of a part of the substrate or product that has strong affinity for a part of the displayed enzyme (noncovalent connection between part of the enzyme and its substrate; see Figure 2B). Such a method allows direct selection for catalysis and does not require any knowledge of the structure of the transition state or of the mechanism of catalysis.[19–25]

We have developed a novel system that allows the in vivo selection of a catalytically active enzyme. Bothmann and Plückthun selected an active chaperone in vivo using phage display. In their system, misfolded scFv was converted to the correctly folded form by the action of the chaperone.[26] Potential substrates, displayed on a phage, that can be labeled with biotin

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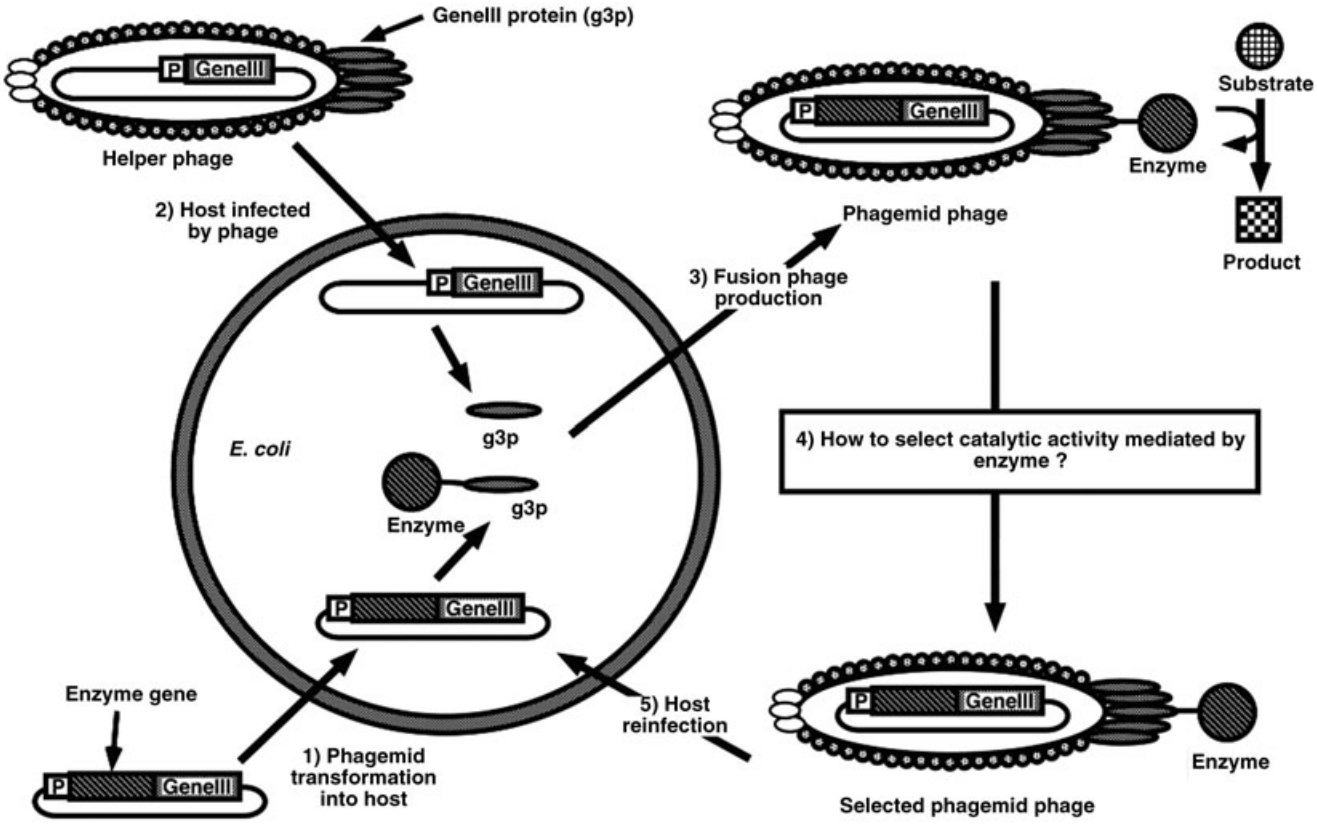

Figure 1. *In vitro selection of enzymes by using phage display. The various steps are described as follows: 1) Phagemid carrying an enzyme library is introduced into* E. coli*. 2) and 3) A pool of phages that display the enzyme is generated by infection of the host cell with helper phage. 4) Enzyme selection on the basis of its catalytic activity is difficult since, for example, reaction products diffuse away from the enzyme after catalysis. 5) Selected phage is used to infect* E. coli*, and the cycle of amplification and selection is repeated. P = promoter site.*

have also been selected in vivo by a phage display system.[27] In our system for in vivo selection of active enzymes, substrate- and enzyme-coding DNA are both introduced into *Escherichia coli* (Figure 3). An engineered M13 filamentous phage, namely the substrate-displaying phage, in which the DNA sequence that encodes the substrate has been fused upstream of gene III (*g3*) is used to infect *E. coli*. The *E. coli* harbors a phagemid with a DNA sequence library that encodes proteins (protein library). In the host-cell cytoplasm, the substrate that is fused to the gene III protein (g3p) is converted to the product if a protein encoded by the library catalyzes the appropriate reaction.[6] Then, the product, which is fused to g3p, is displayed on the surface of the phagemid-phage. When the phagemid-phages are selected on the basis of product affinity, it is possible to isolate those that have packaged the genome that encodes the protein with catalytic activity. The cycle of amplification and selection can be repeated with the selected phagemid-phage.

Our novel method for selection of enzymes has the advantages of protein selection both in vivo and in vitro. The properties of the selected enzyme reflect the cellular environment because the enzyme catalyzes the reaction in the cell. Moreover, because the phage display method is used, the diversity of protein libraries is larger than that associated with other in vivo methods. Finally, the size of the enzyme is not a limiting factor, because as it acts in the *E. coli* cytoplasm, it is not displayed on the surface of the phage.

Results

Construction of a system for the selection of an active biotin protein ligase (BPL) in vivo by using phage display

The goal of this study was to establish a method for the selection of enzymes on the basis of their catalytic activity in vivo by using phage display. To confirm the validity and potential utility of the method that we have developed, we used *E. coli* BPL as a model enzyme (Figure 3).[27–29] BPL catalyzes the formation of biotinyl-5′-adenylate from biotin and ATP, and transfers biotin to a specific lysine residue on the biotin carboxyl carrier protein (BCCP), which is a subunit of acetyl-CoA carboxylase. The biotinylation reaction is highly specific and only biotin-dependent carboxylases, including acetyl-CoA carboxylase, serve as substrates in vivo.[30,31]

Firstly, for the infection of phages into cells, we generated the *E. coli* strain BM4062F′, which we produced by introducing an F′ plasmid (*tet*′) into BM4062 cells that harbor a temperature-sensitive *birA* gene as a result of an Arg235Cys point mutation.[27–29,32] The generated BM4062F′ cells have both F′ plasmid and the endogenous mutant *birA* gene that expresses

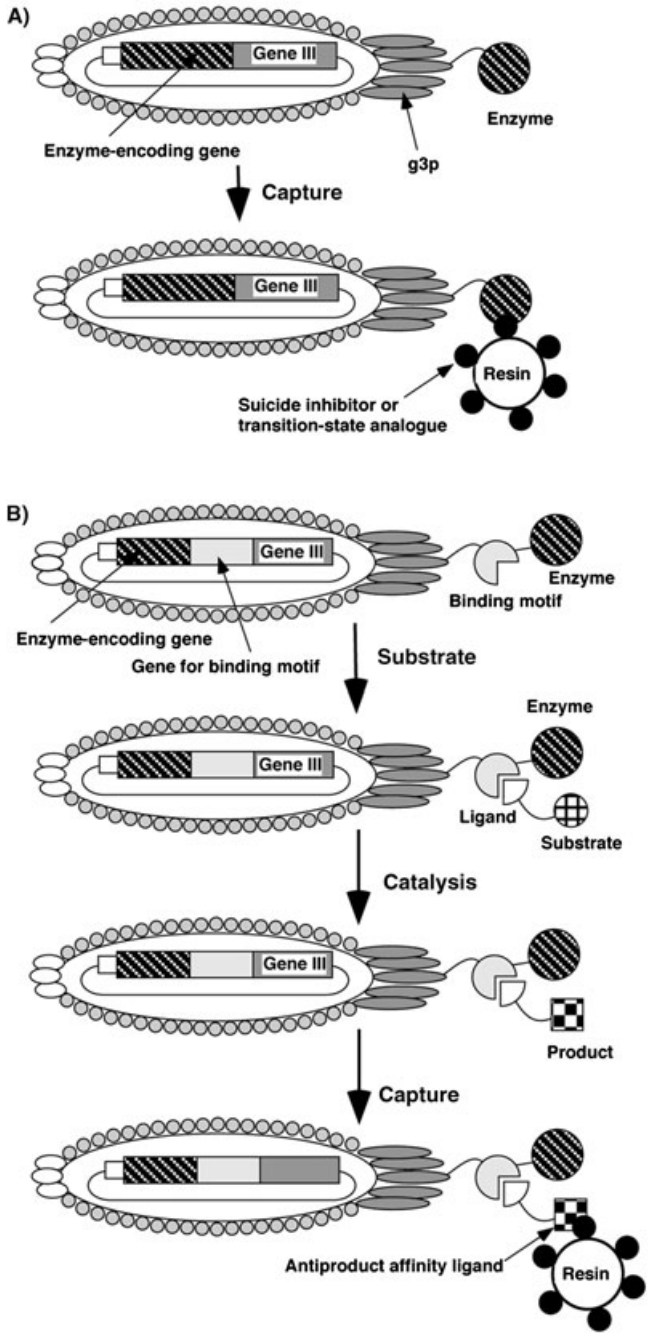

Figure 2. *A) The selection of an enzyme displayed on phage by chemical linkage to a suicide inhibitor or to a transition-state analogue that is covalently connected to a solid support. B) Selection of an enzyme by display of both enzyme and substrate on phage.*

mutant BPL. The mutant BPL has very low activity at 37 °C and requires a high concentration of biotin for the biotinylation reaction.

The enzyme-coding phagemid, pTVBirA, that we prepared carried the *birA* gene. For the negative-control phagemid, pTVlacZα, we replaced the *birA* gene of pTVBirA by the *lacZα* gene, the product of which is the α-peptide derived from β-galactosidase. Both pTVBirA and pTVlacZα carried an ampicillin-resistance gene (*amp*^{r}) and included the origin of replication of the pUC plasmid. The levels of expression of the *birA* and *lacZα* genes were regulated by the lactose promoter (P_{lac}) and the lactose operator (*lacO*), so that both genes were inducible by isopropyl-1-thio-β-D-galactoside (IPTG).

We prepared M13KE-Btag-phage as the substrate-encoding phage, since a 14-residue peptide, the biotin-tag-peptide (Btag), was recently identified by combinatorial methods as the minimum sequence required by the *E. coli* BPL.[33] M13KE-Btag-phage was a simple derivative of M13KE, in which the sequence that encodes Btag had been introduced between the signal sequence and the 5′ end of *g3*. We anticipated that Btag, when fused to the amino terminus of g3p and thus giving rise to the Btag-g3p fusion protein, would be incorporated into the phage capsid (Figure 3).

In this study, as mentioned above, we used *E. coli* BM4062F′ as the host for selection because repression of the activity of endogenous BPL is necessary to minimize background biotinylation of Btag. *E. coli* BM4062F′ was transformed with either pTVBirA or pTVlacZα, and then host cells were incubated in LB medium with biotin, ampicillin, and tetracycline. Then the pTVBirA- or pTVlacZα-harboring cells were infected with M13KE-Btag-phage, and incubation was continued.

We anticipated that the Btag-g3p fusion protein, expressed from the genome of the M13KE-Btag-phage, should be labeled with biotin if, and only if, active BPL had successfully catalyzed biotinylation in the host cells. In theory, the progeny phagemid-phages that have packaged the pTVBirA genome (pTVBirA-phage) and the progeny phages that have packaged M13KE-Btag should both display the biotinylated Btag-g3p fusion protein, but only the former should include the genetic information for synthesis of the enzyme. In practice, both the avidin-bound biotinylated-pTVBirA phage and the avidin-bound biotinylated-M13KE-Btag-phage were collected and used to reinfect host cells (step 6 in Figure 3). However, only host cells infected with biotinylated pTVBirA-phage were able to survive on plates prepared with ampicillin because only the pTVBirA-phage carried the *amp*^{r} gene. This phenomenon allowed for selection based on the catalytic activity of BPL.

Confirmation of in vivo biotinylation of Btag

To confirm the in vivo biotinylation of Btag that was displayed on phagemid-phage under the above-described conditions, we infected *E. coli* BM4062F′ that harbored either pTVBirA or pTVlacZα, or a mixture of *E. coli* that harbored either of the phagemids at a ratio of 1:1 or 1:10, with M13KE-Btag-phage (steps 1 and 2 in Figure 3). In this study, biotinylation of phagemid-phage was carried out at a "leaky" level of BPL and biotin normally present in commercial LB medium, without further addition of biotin (see Supporting Information and Figure S1). We purified the mixture of each secreted phagemid-phage and phage after incubation at 37 °C for 4.5 h or at 30 °C for 16 h. In the case of *E. coli* that harbored pTVBirA, we anticipated that both biotinylated phagemid-phage particles, which included the *birA* gene, and biotinylated phage particles, which included the gene for the substrate, would be secreted by the host cells (step 3 in Figure 3). Similarly, in the case of host cells that har-

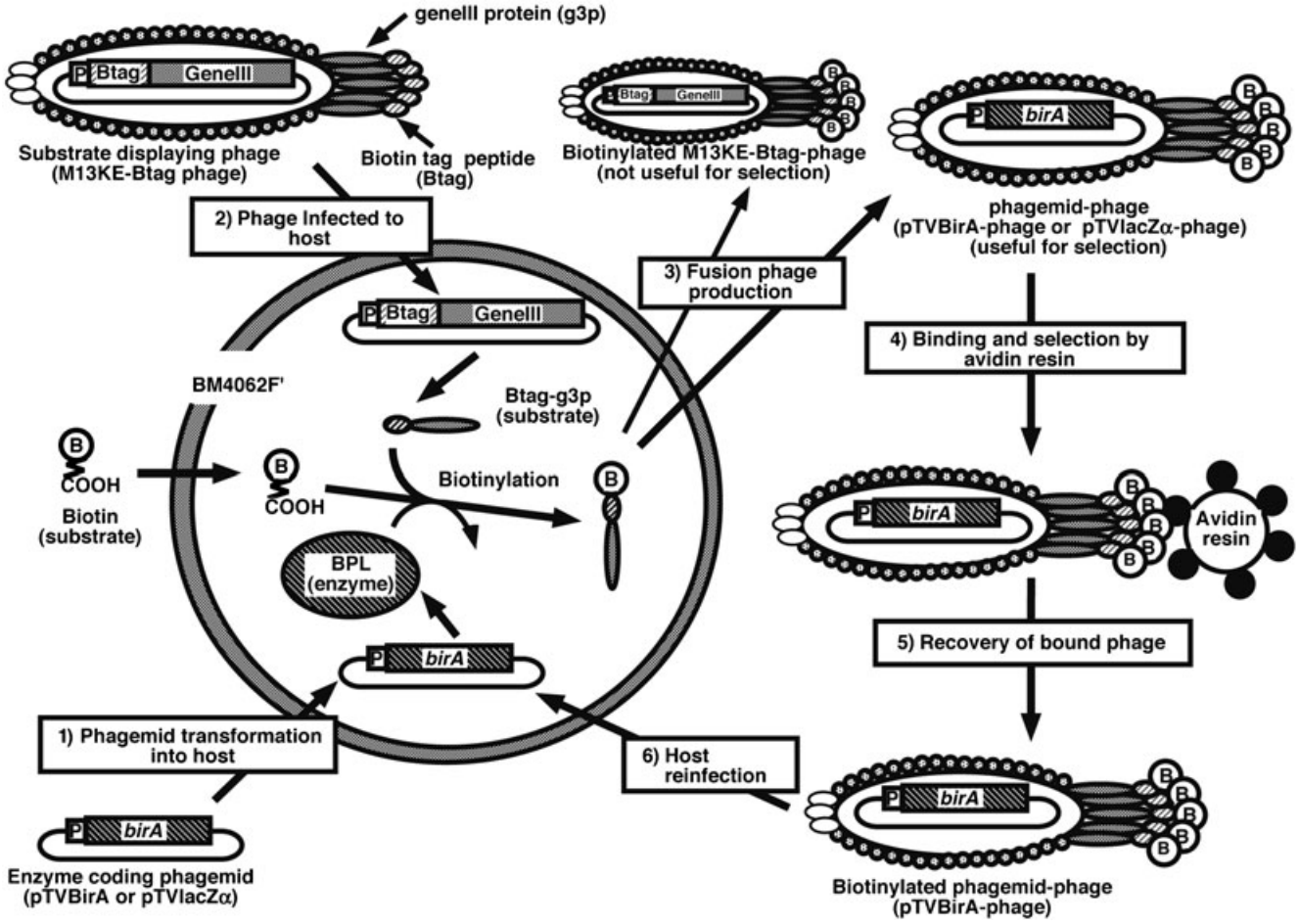

Figure 3. *Schematic representation of the in vivo selection system for an enzyme on the basis of catalytic activity by using phage display. The steps for the selection of BPL from a pool that contains BPL and α-peptide, derived from β-galactosidase, are as follows. 1) A library containing phagemids that include the* birA *gene or the* lacZα *gene is introduced into* E. coli *BM4062F′ host cells. 2) The host cells are then infected with M13KE-Btag-phage. 3) A mixture of phagemid-phage and phage that display the Btag peptide is generated. If BPL is expressed in* E. coli*, Btag is biotinylated. 4) Biotinylated phage particles are captured on avidin resin. 5) and 6) After washing, phage particles that have bound to the resin are used to infect* E. coli *and the cycle of amplification and selection is repeated. P = promoter site.*

bored pTVlacZα, both phagemid-phage particles that included the *lacZα* gene and phage particles that included the gene for the substrate would be secreted without biotinylation from the cells. Thus, we reasoned that, in the case of a mixture of *E. coli* cells that harbored pTVBirA and pTVlacZα, four kinds of particle would be secreted: biotinylated phagemid-phage, biotinylated phage, phagemid phage-without biotinylation, and phage without biotinylation (step 3 in Figure 3). Titers of phage and phagemid-phage particles could be determined after purification by counting plaques and colonies, respectively.

To confirm the efficiency of in vivo biotinylation of Btag, we mixed each purified sample of 10^6 phagemid-phages with SoftLink™ soft-release avidin resin, which carries immobilized monomeric avidin that binds to biotin with a K_d of 10^{-7} M and allows the elution of biotinylated phage under mild conditions, for example, by the addition of biotin as a competitor (steps 4–5 in Figure 3). As a control for measurement of background levels, we treated the resin with excess biotin prior to the addition of the purified sample so that even biotinylated phage would not bind to the resin (black bars in Figure 4A and B).

The material that had bound to the resin, which included phagemid-phage, was eluted by a solution of biotin (step 5 in Figure 3), and the eluate was mixed with *E. coli* ER2738 for infection by phagemid-phage (step 6 in Figure 3). The cells were then plated on agar-solidified LB medium that contained ampicillin and tetracycline. Since the cells, which carried the *tet*^{r} gene, had been infected with phagemid that included the *amp*^{r} gene, they were able to survive on such plates. We were then able to monitor the recovery efficiency of each phagemid-phage by counting colonies. Our results revealed that at either 37 °C or 30 °C, the number of ER2738 colonies infected with pTVBirA-phage was six to ninefold higher than that of ER2738 cells infected with pTVlacZα (Figure 4A and B).

At 37 °C, 17% of the pTVBirA-phages was recovered from the resin, whereas only 2% (12% relative to pTVBirA-phage) of pTVlacZα-phages was recovered, as shown by shaded bars in Figure 4A. The efficiency of isolation of pTVlacZα-phages was

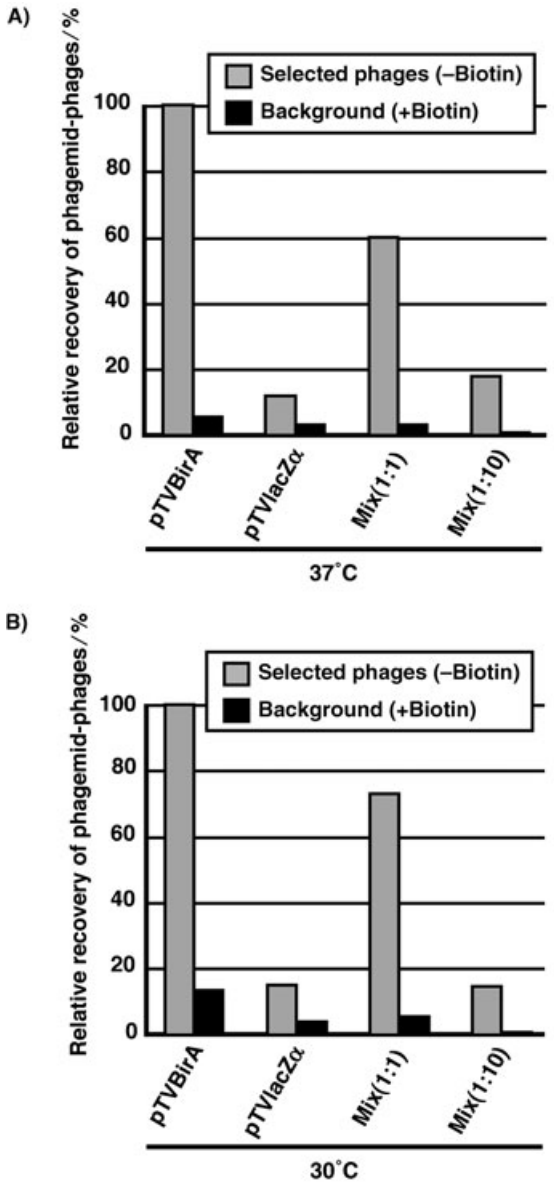

Figure 4. *The efficiency of recovery of individual phagemid-phage. Results are averages from two or three independent experiments.* E. coli *BM4062F′ that harbored pTVBirA, pTVlacZα, or pTVBirA and pTVlacZα at a ratio of 1:1 or 1:10 were infected with M13KE-Btag-phage. After incubation at 37°C for 4.5 h or at 30°C for 16 h, released phage particles were purified and allowed to bind to avidin resin. The numbers of phagemid-phages are indicated in relation to the number of pTVBirA-phages. A) Relative recovery of phagemid-phages at 37°C. The recovered pTVBirA-phages were 16.7% of the total input. B) Relative recovery of phagemid-phages at 30°C. The recovered pTVBirA-phages were 12.3% of the total input.*

higher than the efficiency of the nonspecific binding of pTVlacZα-phages to the resin after prior treatment of the resin with excess biotin (black bars in Figure 4A and B). Thus, it is likely that some pTVlacZα-phages had been biotinylated by endogenous BPL even though we had used *E. coli* BM4062F′ cells as host. We anticipated that, when we mixed *E. coli* cells that harbored pTVBirA or pTVlacZα at a ratio of 1:1 and 1:10, the recovery of phagemid-phages compared to the recovery of pTVBirA-phages, which were generated from *E. coli* cells that harbored only pTVBirA, in the absence of background biotinylation would be $^1/_2$ (50% of biotinylated pTVBirA-phages and 50% of pTVlacZα-phages without biotinylation) and $^1/_{11}$ (9% of biotinylated pTVBirA-phages and 91% of pTVlacZα-phages without biotinylation), respectively. Therefore, the actual recovery ratios of 60% and 18% (37°C), or 73% and 15% (30°C) are reasonable (Figure 4A and B).

Our results showed that, during culture at 37°C and at 30°C, the efficiency of biotinylation by endogenous mutant BPL was very low, so that pTVBirA-phage was selectively biotinylated by BPL that was expressed from pTVBirA.

Selective enrichment of the phagemid-phages that packaged the pTVBirA genome

As described above, we qualitatively confirmed the successful biotinylation of the substrate by using BPL encoded by pTVBirA. We next tried to enrich our preparation for the enzyme-coding phagemid-phage and to quantitate the exact level *birA* gene enrichment by colony PCR. In each selection cycle, we recovered a sample that included phagemid-phage that had bound to the resin (step 5 in Figure 3). This sample was mixed with *E. coli* ER2738 for the reinfection of cells with phagemid-phage (step 6) and the infected cells were plated on agar-solidified LB medium that contained ampicillin and tetracycline. Only tetracycline-resistant cells that had been infected with phagemid that encoded *amp*^{r} survived on such plates. The surviving *E. coli* cells were then infected with phage (step 2) for the next round of selection (step 3) and further enrichment.

In this study, to confirm that the pTVBirA and pTVlacZα libraries could indeed be selectively enriched for the pTVBirA genome by using our system, we investigated whether pTVBirA- or pTVlacZα-phagemids were included in the *E. coli* cells that survived on the above-mentioned ampicillin plates (step 6). In this specific case, we identified pTVBirA- or pTVlacZα-phagemids by using colony PCR (Table 1). To confirm

Table 1. *Selective enrichment for pTVBirA-phages that displayed the Btag-g3p fusion protein*

Conditions[a]	Before selection[b]		After selection[c]		Enrichment factor
	pTVBirA	pTVlacZα	pTVBirA	pTVlacZα	
(1:1)	19	13	13	3	3.0
(1:1)	11	9	15	5	2.5
(1:1)	11	9	18	2	7.4
(1:10)	4	20	16	8	10.0
(1:10)	0	20	6	14	$\geq$8.6
(1:10)	0	20	3	17	$\geq$3.5

[a] A mixture of *E. coli* BM4062F′ cells that harbored pTVBirA or pTVlacZα at a ratio of 1:1 or 1:10 were infected with M13KE-Btag-phage (4×10^9 pfu). The infected cells were incubated in LB medium at 37°C for 4.5 h. [b] The ratio of the number of secreted pTVBirA-phages to the number of secreted pTVlacZα-phages. Even if pTVBirA and pTVlacZα were transformed to *E. coli* at a ratio of 1:1 or 1:10, the ratio of pTVBirA-phages to pTVlacZα-phages prior to selection is not always 1:1 or 1:10 in each experiment. [c] The ratio of the number of pTVBirA-phages to that of pTVlacZα-phages after selection.

the ratio of pTVBirA- to pTVlacZα-phage before selection with avidin (prior to step 4) we also examined which phagemid, namely pTVBirA or pTVlacZα, was included in the surviving *E. coli* cells on the ampicillin plates that had been prepared for determination of the titer of each secreted phagemid-phage (see previous section).

We found that, at 37 °C, the concentration of pTVBirA-phage was successfully increased 2.5 to tenfold from the pool of *E. coli* cells that harbored pTVBirA or pTVlacZα at a ratio of 1:1 or 1:10 (Table 1). In the case of each result shown in Table 1, the enrichment (2.5- to tenfold) cycle was performed only once. At 30 °C, pTVBirA-phage was also enriched 2.5 to tenfold from the pool of *E. coli* cells (data not shown). When the concentration of the desired enzyme-coding phagemid is low, repeated rounds of selection should allow easy isolation of the desired phagemid. In our experiments, we failed to eliminate the background activity of endogenous BPL completely. It should be possible to improve the selection system that is based on enzymatic activity if the system is based on an enzyme, such as an acetylase, Dicer, or caspase, that is not encoded by the *E. coli* genome.

Discussion

In this study, we developed a method for in vivo selection of enzymes that is based on catalytic activity using phage-display. Our method has several advantages over earlier phage-display methods. i) Since the substrate is converted to the product by an enzymatic reaction in *E. coli*, the properties of the selected enzyme reflect the cellular environment. Thus, we automatically select enzymes that are stable in vivo. ii) Since the enzyme is expressed and catalyzes a reaction in *E. coli* without secretion from cells, the size and nature of the enzyme, which might influence phage secretion, are not limiting factors. iii) In general, M13 is not a useful vehicle for the expression of cDNAs, because of the requirement for in-frame connection with both the leader sequence, which is required for secretion, and the amino terminus of the coat protein, g3p. Thus, the DNA must be inserted in the correct reading frame at both ends ($p = 1/9$). This requirement results in a vanishingly small number of productive clones in M13 cDNA libraries. However, in our method, cDNAs have to be inserted in the correct reading frame only at the amino terminus ($p = 1/3$) because the enzyme has only to catalyze a reaction in *E. coli* and does not have to be displayed on the surface of the phage. Therefore, the number of productive clones is threefold higher than that in the conventional phage-display method.

Using our method for enzyme selection, in theory we should be able to select enzymes that catalyze modification of peptides or proteins, such as protein ligase, acetylase, kinase, phosphatase, ubiquitinase, and protease (including caspase). The in vivo selection of a chaperone and a substrate sequence by using phage has already been reported, but this report provides the first evidence that an enzyme can be selected in vivo by using this method. Our system should help us to analyze mechanisms of signal transduction and to isolate unknown enzymes encoded by cDNA libraries, as well as artificial enzymes.

Experimental Section

Construction of phagemids, phage, and host strain of *E. coli*: The commercially available phagemid pTV118N (Takara, Kyoto, Japan) is renamed pTVlacZα in this paper to indicate the presence of the *lacZα* gene. pTV118N has an ampicillin-resistance gene (*amp*^{r}), the origin of replication of plasmid pUC, and a *lacZα* gene that is regulated by a lactose promoter (P_{lac}) and operator (*lacO*). Phagemid pTVBirA was derived from pTV118N as follows. The *birA* gene[27,29–31,34] which encodes BPL was amplified from the genome of *E. coli* ER2738 (New England BioLabs, Beverly, MA, USA) and inserted downstream of *lacO* in pTV118N to replace the *lacZα* gene.

The M13KE-Btag-phage was derived from M13KE-phage (New England BioLabs) as follows. i) *E. coli* ER2738 was infected with M13KE and ii) the replicative form (RF) of the phage that was generated in *E. coli* was purified with a QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany); iii) A DNA oligonucleotide encoding a 14-residue peptide (GLNDIFEAQKIEWH),[33] which we call Btag, was synthesized and ligated between the signal sequence and *g3* in the RF of M13KE. The purified RF was used to transfect *E. coli* and the M13KE-Btag-phage released from the cells was purified.

E. coli BM4062F′, derived from *E. coli* BM4062, harbor an F′ plasmid that includes a tetracycline-resistance gene (*tet*^{r}).[27–29,32] *E. coli* ER2738 harboring the F′ plasmid that included *tet*^{r}, was mixed with BM4062 cells that carried a streptomycin-resistance gene (*str*^{r}) and the mixture was incubated in Luria–Bertani (LB) medium for 1 h at 37 °C. The mixture was then plated on agar-solidified LB medium with streptomycin (50 mg mL^{-1}) and tetracycline (50 mg mL^{-1}). Surviving cells were isolated as *E. coli* BM4062F′.

Generation of the phagemid-phage: We incubated BM4062F′ cells that harbored pTVBirA or pTVlacZα, in LB medium (8 mL) at 37 °C or 30 °C with ampicillin (50 mg mL^{-1}), tetracycline (50 mg mL^{-1}), and ATP (1 mM). At the logarithmic phase of growth (OD at 600 nm ≈0.6), we mixed the cultures to generate a mixture of *E. coli* that harbored pTVBirA or pTVlacZα at a ratio of 1:1 or 1:10. These cells were infected with M13KE-Btag-phage (4×10^9 pfu). After incubation at 37 °C for 4.5 h or 30 °C for 16 h, released phage and phagemid-phage were purified by precipitation with polyethylene glycol. Samples were stored in TBS buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl).

Affinity selection of phagemid-phage that display the biotinylated Btag peptide: A mixture of 10^6 phagemid-phage (pTVBirA-phage, pTVlacZα-phage, or a mixture of pTVBirA- and pTVlacZα-phages at ratio or 1:1 or 1:10) and 10^6 phage (M13KE-Btag-phage) in TBST buffer (50 μL; 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 % Tween 20) was added to SoftLink™ soft-release avidin resin (7 μL; Promega, Madison, WI, USA) with or without biotin (1 mM). After incubation for 1 h at 30 °C, the resin was washed with TBST buffer (3 × 1 mL). For elution of the phage, elution buffer (100 μL; 100 mM sodium phosphate buffer (pH 7.0), 5 mM biotin) was added to the resin. Then elution buffer (1 μL) that contained the selected phagemid phage was added to LB medium (100 μL) that contained *E. coli* ER2738 at the logarithmic phase of growth (OD at 600 nm ≈0.6). After incubation for 10 min at room temperature, the mixture was plated on agar-solidified LB medium with ampicillin (50 mg mL^{-1}) and tetracycline (50 mg mL^{-1}) to determine the titer of the selected phagemid-phage.

In order to determine which phagemid, pTVBirA or pTVlacZα, had infected the cells, each surviving colony of *E. coli* cells that harbored phagemids that included the *birA* or the *lacZα* gene was used as template for the PCR reaction mixture. PCR products were

then analyzed by electrophoresis on an agarose gel and the phagemids in the cells identified. Enrichment factor was calculated by the following equation:

$$\text{Enrichment factor} = \frac{\text{pTVBirA after selection}}{\text{pTVlacZ}\alpha\text{ after selection}} \times \frac{\text{pTVBirA before selection}}{\text{pTVlacZ}\alpha\text{ before selection}}$$

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