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CHEMICAL COMPLEMENTATION: A REACTION-INDEPENDENT GENETIC ASSAY FOR DIRECTED EVOLUTION

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INTRODUCTION

Enzymes are able to catalyse a broad range of chemical transformations not only with impressive rate enhancements but also with both regio- and stereo-selectivity and so are attractive candidates as practical alternatives to traditional small molecule catalysts. With applications as diverse as chemical synthesis, reagents for commercial products and biomedical research, and even therapeutics, there is a great demand for enzymes with both improved activity and novel catalytic function [1,2]. In theory, the properties of an enzyme can be altered by rational design; however, rational design is greatly hindered in practice by the complexity of protein function. With advances in molecular biology the possibility has arisen that an enzyme with the desired catalytic property can instead be isolated from a large pool of protein variants. Recently directed evolution has been used successfully to modify the substrate [3] or cofactor specificity [4] of an existing enzyme. These experiments, however, are limited to reactions that are inherently screenable or selectable-reactions where the substrate is a peptide [6,7] or the product is fluorescent [4] or an essential metabolite [3,8,9]. What is needed now to realize the power of directed evolution experiments are screening and selection strategies that are general-strategies that do not limit the chemistry and that can readily be adapted to a new target reaction.

Early success with assays based on binding to transition-state analogues and suicide substrates convinced researchers that it should be possible to engineer proteins to catalyse a broad range of reactions [10], but it was difficult to translate binding events into read-outs for enhanced catalytic activity. Recently, attention has turned to direct selections for catalytic activity. While strategies ranging from *in vitro* fluorescence assays to physically linking the enzyme to its substrate have all recently been reported [10-15], a general solution to this problem is yet to emerge. *In vivo* complementation, in which an enzyme is selected based on its ability to complement an essential activity that has been deleted from a wild-type cell, has proven to be one of the most powerful approaches to enzyme evolution [3,5,8]. However, complementation assays are limited to natural reactions that are selectable. In this paper we describe our efforts to develop a "chemical complementation" system, which would allow us to control the chemical reaction linked to cell survival, extending complementation approaches to a broad range of chemical transformations.

RESULTS AND DISCUSSION

Strategy

Our approach to developing such a chemical complementation system is to use the yeast three-hybrid assay to link enzyme catalysis to transcription of a reporter gene *in vivo* (Fig. 1).

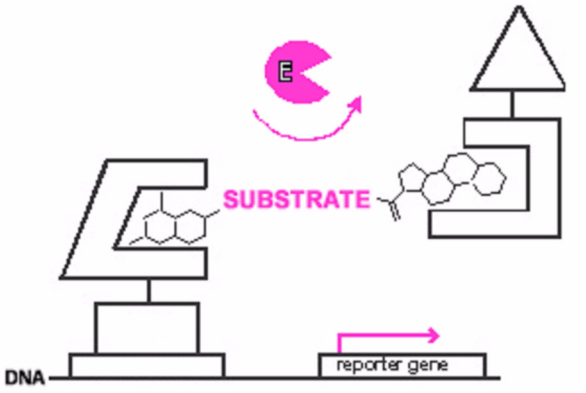


Figure 1. Chemical Complementation. A reaction-independent complementation assay for enzyme catalysis based on the yeast three-hybrid assay. A heterodimeric small molecule bridges a DNA-binding domain-receptor fusion protein and an activation domain-receptor fusion protein, activating transcription of a downstream reporter gene *in vivo*. Enzyme catalysis of either cleavage or formation of the bond between the two small molecules can be detected as a change in transcription of the reporter gene. The assay can be applied to new chemical reactions simply by synthesizing small molecules with different substrates as linkers and adding an enzyme as a fourth component to the system.

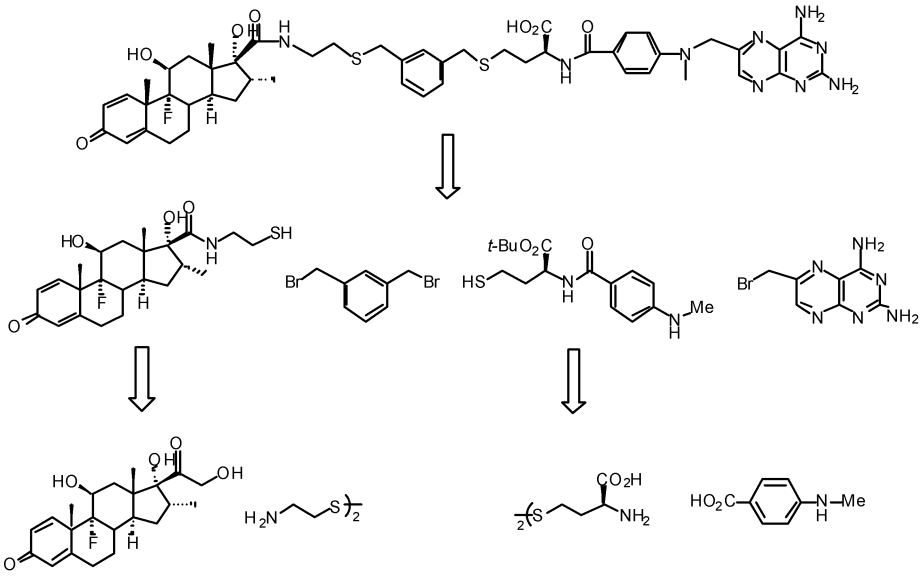
As a first step, we designed a yeast three-hybrid system in which a dimeric small molecule bridges a DNA-binding domain-receptor fusion protein and an activation domain-receptor fusion protein. By dimerizing the two fusion proteins via the receptors, the small molecule effectively reconstitutes the transcriptional activator, turning on transcription of a downstream reporter gene. We envisioned that this system could be used as a read-out for enzyme catalysed bond cleavage or formation reactions simply by replacing the chemical linker between the two small molecules with the bond to be cleaved or formed and adding an enzyme as a fourth component to the system. Cleavage of the bond between the two small molecules by an active enzyme would disrupt reconstitution of the transcriptional activator and, hence, transcription of the reporter gene. Bond formation would activate transcription of the reporter gene. This approach is general because it can be applied to new reactions simply by synthesizing heterodimers with different substrates as chemical linkers. Genetic assays are high-throughput because large pools of proteins can be sorted based on clear changes in cellular phenotype. We then developed the chemical complementation system around the well-studied reaction, cephalosporin hydrolysis by a cephalosporinase (β-lactam hydrolase, EC 3.5.2.6). Using this model system, we went on to show that the catalytic efficiency (k_{cat}/K_M) of cephalosporinase variants correlates with their levels of transcription activation in the three-hybrid assay. In preliminary results, we have now demonstrated the generality of this approach, applying it to the directed evolution of an enzyme that catalyses glycosidic bond formation.

Three-Hybrid System [16]

The first step then was to design a three-hybrid system in which dimerization of a transcriptional activator is dependent on a heterodimeric small molecule. For this work, we built from a dexamethasone-FK506 yeast three-hybrid system reported by Licitra and Liu [17] and precedent from the Schreiber and other laboratories in the design of chemical inducers of dimerization (CIDs) [18,19]. We chose to build a heterodimeric CID based on the well-characterized ligand-receptor pairs dexamethasone (Dex)-glucocorticoid receptor (GR) and methotrexate (Mtx)-dihydrofolate reductase (DHFR). Both Dex and Mtx present chemical functionality that can be modified readily without disrupting receptor binding. The rat glucocorticoid receptor (rGR) binds Dex with a KD of 5 nM, and mutants of rGR with increased affinity for Dex have been isolated [20].

The steroid dexamethasone has been used extensively as a cell-permeable small molecule to regulate the activity and nuclear localization of GR fusion proteins *in vivo* [21]. Dex is commercially available and can be derivatized at the C-21 position without disrupting its binding to GR [22,23]. Likewise, the interaction between Mtx and DHFR is extremely well characterized both biochemically and structurally [24,25]. DHFR fusion proteins have been used for a variety of biochemical applications due to Mtx's picomolar K_I for DHFR [26]. Mtx is cell permeable, commercially available, and can be synthesized readily from simple starting materials.

Scheme 1



The retro-synthetic analysis of the Dex-Mtx heterodimer is shown in Scheme 1. The synthesis is based on previous syntheses of Dex and Mtx derivatives [22,23]. The synthesis is designed to allow the chemical linker between the two ligands to be varied readily. Both ligands were introduced as thiol derivatives to a di-halo linker. Following oxidative cleavage with periodate, Dex was dervatized with cystamine using standard peptide coupling reagents.

The γ-carboxylate in Mtx was replaced with a thiol simply by replacing glutamate with homocysteine. Homocysteine, protected as the tert-butyl ester and disulfide, was coupled to 4-methylaminobenzoic acid. The resulting Dex and Mtx disulfide derivatives were reduced to their corresponding thiols using tri-n-butylphosphine, and the two thiols were coupled to a di-bromo linker in a one-pot reaction. The 2,4-diamino-6-bromomethyl pteridine was added after introduction of the dibromo linker to simplify purification of the intermediates, and the final step was cleavage of the tert-butyl ester.

Using a modification of this original synthesis, the Dex-Mtx heterodimer is now routinely prepared from two components in 6 steps in 10% overall yield. The modular synthesis of the Dex-Mtx heterodimer was designed with the chemical complementation system in mind, where the linker between the two halves of the molecule will need to be varied.

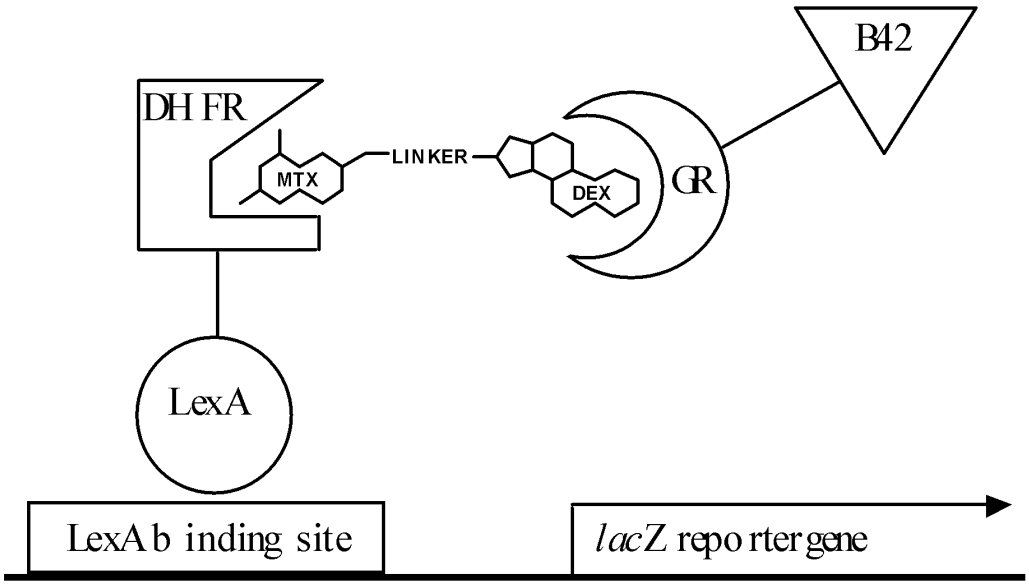


Figure 2. Yeast three-hybrid assay. A binding site with high affinity for the DNA-binding protein LexA is placed upstream of a *lacZ* reporter gene, which encodes β-galactosidase. The LexA-DHFR protein chimera binds to the LexA binding site. In the presence of the Dex-Mtx CID, the LexA-DHFR chimera binds to Dex-Mtx which in turn binds to the GR-B42 protein chimera. The Dex-Mtx CID, thus, effectively brings the transcription activation protein B42 to the LexA binding site. Once bound to the LexA binding site, B42 recruits the transcriptional machinery to the *lacZ* reporter gene, and β-galactosidase is synthesized. The levels of β-galactosidase synthesis, therefore, are a good indicator of the efficiency of Dex-Mtx-induced protein dimerization.

For the genetic assay, we chose the yeast "three-hybrid" system [17,28,29], which consists of a dimeric small molecule, two protein chimeras, and a reporter gene (Fig. 2). The small molecule (Dex-Mtx) bridges the DNA-binding protein chimera (LexA-DHFR) and the transcription activation protein chimera (B42-GR). Dimerization of LexA and B42 recruits the transcription machinery to the promoter near the LexA-binding site, thereby activating transcription of the *lacZ* reporter gene. Plasmids encoding the LexA-DHFR and B42-GR protein chimeras were prepared from the Brent two-hybrid vectors pMW102 and pMW103 using standard molecular biology techniques [30,31]. Plasmid pMW106, which encodes the *lacZ* reporter gene under control of four tandem LexA operators, was used as provided. All three plasmids were introduced into *S. cerevisiae* strain FY250, and the resultant strain was used to test the activity of the Dex-Mtx CID.

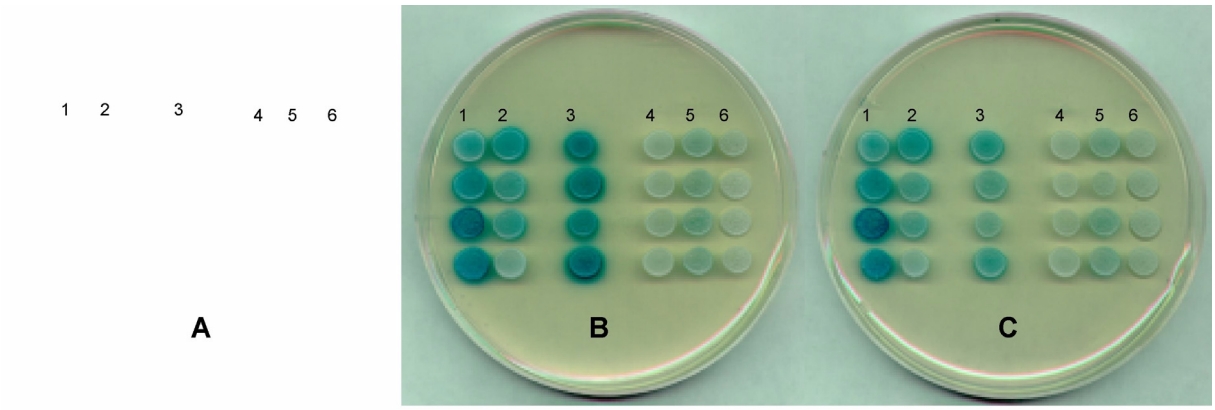


Figure 3. X-gal plate assay of Dex-Mtx-induced *lacZ* transcription. Yeast strains containing a *lacZ* reporter gene and different LexA- and/or B42-chimeras were grown on X-gal indicator plates with or without Dex-Mtx. Columns **1-6** on each plate correspond to yeast strains containing different LexA- and/or B42-chimeras: **1)** LexA-Sec16p, B42-Sec6p; **2)** LexA-Sec13, B42-Sec6p. 1 and 2 are direct protein-protein interactions used as positive controls [27] ; **3)** LexA-DHFR, B42-GR; **4)** LexA-DHFR, B42; **5)** LexA, B42-GR; **6)** LexA, B42. X-gal plates **A-C** have different small molecule combinations: **A)** no Dex-Mtx; **B)** 1 μM Dex-Mtx; **C)** 1 μM Dex-Mtx, and 10 μM Mtx.

Using standard b-galactosidase activity assays both on plates and in liquid culture [31] we showed that Dex-Mtx could activate *lacZ* transcription *in vivo* (Fig. 3 and data not shown). Based on previous studies showing that *lacZ* transcription levels correlate with the strength of protein-protein interactions in the yeast two-hybrid assay, we expect β-galactosidase activity to be a good indicator of Dex-Mtx induced protein dimerization. In these assays, the extracellular concentration of Dex-Mtx ranged from 0.01 to 10 μM. No activation was observed at concentrations < 0.1 μM. Control experiments established that *lacZ* transcription was dependent on Dex-Mtx (Fig. 3 and data not shown). Only background levels of β-galactosidase activity were detected when Dex-Mtx was omitted. A 10-fold excess of Mtx reduced Dex-Mtx-dependent *lacZ* transcription to near background levels. A 10-fold excess of Dex, however, did not affect Dex-Mtx-dependent *lacZ* transcription, and higher concentrations of Dex were toxic to the yeast cells.

This result may be due to differences in cell permeability between Dex and Dex-Mtx or may suggest that LexA-DHFR, but not B42-GR, is the limiting reagent. To provide further support that *lacZ* transcription is Dex-Mtx-dependent, LexA-DHFR was replaced with LexA, or B42-GR was replaced with B42. Again, only background levels of *lacZ* transcription were detected in the presence of Dex-Mtx in the yeast cells containing either LexA and B42-GR or LexA-DHFR and B42.

The Dex-Mtx yeast three-hybrid system proved quite robust, providing the framework for subsequent development of the chemical complementation system. The Dex-Mtx heterodimer could be synthesized readily and cheaply, facilitating future incorporation of the substrate bond into this molecule. The Dex-Mtx heterodimer is a strong transcriptional activator, which is essential in the next stage for linking enzyme catalysis to reporter gene transcription. Because the three-hybrid system is the cornerstone for the chemical complementation system, our laboratory has invested significantly in the development and optimization of this assay. We have characterized the system and made improvements [39], notably the development of a bacterial three-hybrid system, which ultimately should allow large libraries to be evaluated because of the high transformation efficiency of *E. coli* [40].

Chemical Complementation [32]

With the Dex-Mtx yeast three-hybrid system in hand, we next set out to develop the chemical complementation system. We chose cephalosporin hydrolysis by the *Enterobacter cloacae* P99 cephalosporinase as a simple cleavage reaction to demonstrate the selection strategy (Fig. 4). Cephalosporins are β-lactam type antibiotics, and cephalosporinases are the bacterial resistance enzymes that hydrolyse and, therefore, inactivate these antibiotics. The cephalosporinase enzyme is well-characterized biochemically and structurally [33,34], and the synthesis of cephem compounds established [35]. We chose to incorporate Mtx and Dex at the C3' and C7 positions, respectively, of the cephem core. Cleavage of the β-lactam bond in cephalosporins results in expulsion of the leaving group at the C3' position, effectively breaking the bond between Mtx and Dex (Fig. 4A). Thus, the Mtx-Cephem-Dex substrate should dimerize the transcriptional activator causing transcription of the reporter gene in the yeast three-hybrid assay. When the cephalosproinase enzyme is expressed, however, the cephem linkage should be cleaved, and protein dimerization and transcription of the reporter gene should be disrupted.

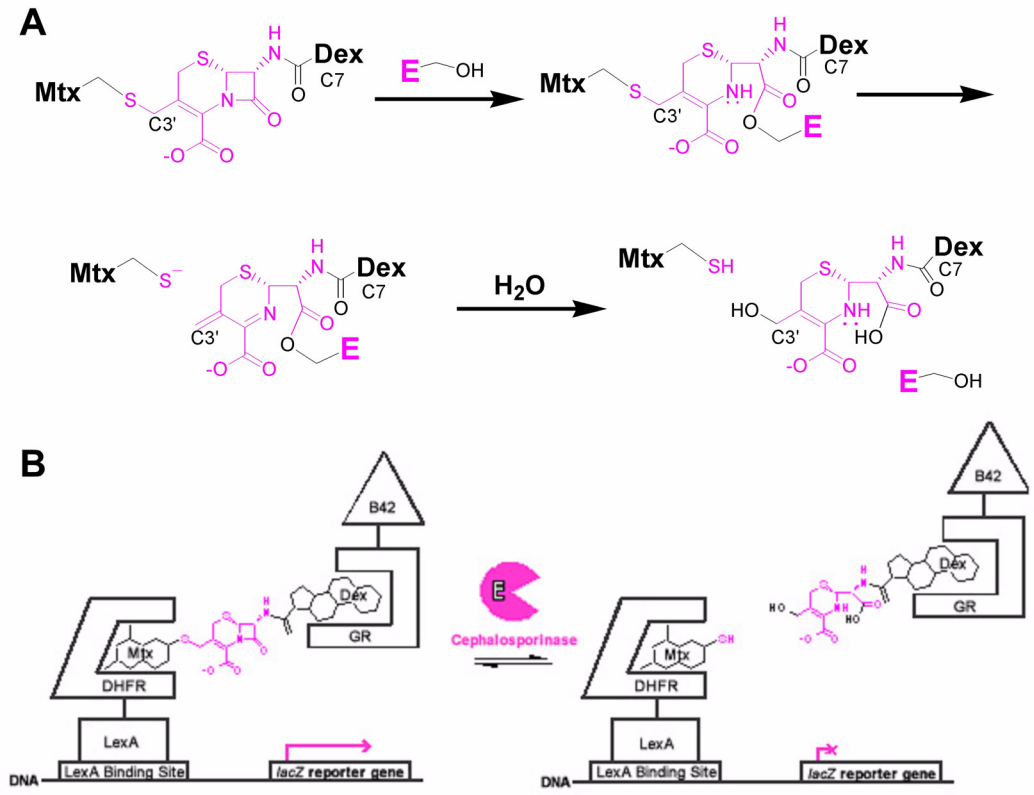


Figure 4. Cephalosporinase Model Reaction. Cephalosporin hydrolysis provides a simple cleavage reaction to demonstrate the complementation strategy. **A)** Cephalosporin hydrolysis by a cephalosporinase enzyme. Cephalosporinases are serine-protease type enzymes and catalyze the hydrolysis of cephalosporin antibiotics. Hydrolysis of the β-lactam bond in Mtx-Cephem-Dex results in expulsion of the leaving group at the C3' position of the cephem core, effectively breaking the bond between Mtx and Dex. **B)** Cephalosporin hydrolysis by the cephalosporinase enzyme disrupts transcription of a *lacZ* reporter gene. The Mtx-Cephem-Dex substrate dimerizes a LexA DNA-binding domain-dihydrofolate reductase (LexA-DHFR) and a B42 activation domain-glucocorticoid receptor (B42-GR) fusion protein, activating transcription of a *lacZ* reporter gene. Addition of active cephalosporinase enzyme results in cleavage of the Mtx-Cephem-Dex substrate and disruption of *lacZ* transcription.

While previous work with the yeast two- and three-hybrid assays suggests that the linker between Mtx and Dex should not affect the transcription read-out, we first wanted to test our assumption that Mtx-Cephem-Dex would retain the ability to activate transcription in our yeast three-hybrid system. In addition, we wanted to carry out *in vitro* kinetic studies to ensure that Mtx-Cephem-Dex is an efficient substrate for the cephalosporinase enzyme. The first step was to synthesize the Mtx-Cephem-Dex substrate. The original synthesis of the Mtx-Dex heterodimer had been designed to facilitate the inclusion of different chemical linkers between the Mtx and Dex portions of the molecule [16]. Thus, the commercial cephem intermediate, 7-amino-3-chlormethyl-3-cephem-4-carboxylic acid p-methoxybenzyl ester (ACLE) could be readily incorporated into this synthesis to prepare Mtx-Cephem-Dex (Fig. 5).

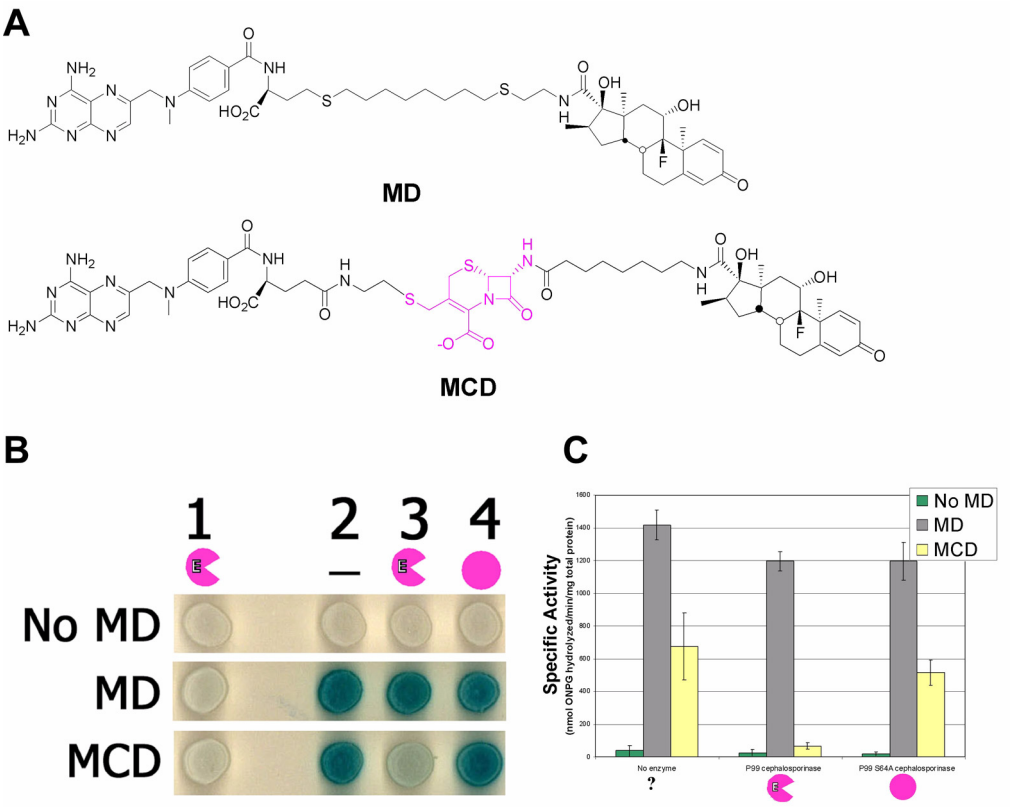


Figure 5. Chemical Complementation Links Enzyme Catalysis to Reporter Gene Transcription. **A)** Structures of the Mtx-Dex (MD) and Mtx-Cephem-Dex (MCD) heterodimers. **B)** X-gal plate assays of cephalosporinase-dependent Mtx-Cephem-Dex-induced *lacZ* transcription. Yeast strains containing a *lacZ* reporter gene were grown on X-gal indicator plates with or without Mtx-linker-Dex molecules as indicated. Columns **1-4** correspond to yeast strains containing a LexA DNA-binding domain fusion protein, a B42 activation domain fusion protein, and enzyme, as follows: **1)** LexA-DHFR, B42, P99 cephalosporinase. 1 lacks GR and is used as a negative control. **2)** LexA-DHFR, B42-GR, no enzyme; **3)** LexA-DHFR, B42-GR, P99 cephalosporinase. **4)** LexA-DHFR, B42-GR, P99 Ser64→Ala cephalosporinase. The rows correspond to individual X-gal plates, which have different small molecules as indicated: **No MD**) No Mtx-Dex; **MD)** 1 µM Mtx-Dex; **MCD)** 10 µM Mtx-Cephem-Dex. **C)** ONPG liquid assays. Yeast strains expressing the LexA-DHFR and B42-GR fusion proteins and containing a *lacZ* reporter gene and expressing either no enzyme, --, P99 cephalosporinase , or P99 Ser^{64}→Ala cephalosporinase, , were grown in liquid culture and assayed for β-galactosidase activity using ONPG as a substrate. The liquid culture contained small molecules as indicated. The assays were done in triplicate. ONPG hydrolysis rates are reported as nmol/min/mg total protein, and the error bars for the specific activity correspond to the standard deviation from the mean. Strains containing the active P99 cephalosporinase showed an 8-fold decrease in the level of *lacZ* transcription relative to strains containing the inactive Ser^{64}→Ala variant.

Using standard β-galactosidase activity assays both on plates and in liquid culture, we showed that Mtx-Cephem-Dex activates *lacZ* transcription in a yeast strain containing the LexA-DHFR and B42-GR fusion proteins and a *lacZ* reporter gene (Figs 5B and 5C, and data not shown). Having shown that Mtx-Cephem-Dex is an efficient dimerizer *in vivo*, we wanted to confirm that it is a good substrate for the cephalosporinase enzyme using purified enzyme *in vitro*.

The P99 cephalosporinase was subcloned into a T7 expression system with a C-terminal His6-tag, over-expressed, and purified using a Nickel-affinity resin. Since Mtx has a strong absorbance at 264 nm, turnover could not be determined based on the change in the absorbance at 264 nm upon cleavage of the lactam bond as is standard [36]. Thus, a coupled assay using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's Reagent) was developed for measuring Mtx-Cephem-Dex hydrolysis. Upon cleavage of the β-lactam bond and expulsion of the Mtx thiol from the C3' position of the cephem, the Mtx thiol reduces Ellman's Reagent, leading to an increase in the absorbance at 412 nm. Using this coupled assay, the P99 cephalosporinase was shown to turnover Mtx-Cephem-Dex with a specific activity of 0.309 ± 0.049 μmol/min/mg of enzyme. By comparison, one of the best substrates for the *E. cloacae* P99 cephalosporinase, nitrocefin, is turned over with a specific activity of 208.7 ± 42.7 μmol/min/mg of enzyme.

Having shown that the Mtx-Cephem-Dex substrate retained the ability to activate transcription *in vivo* and was cleaved effectively by the cephalosporinase enzyme *in vitro*, the next step was to add the enzyme to the yeast three-hybrid system and find conditions where we could observe enzyme-dependent transcription. Towards this end, the yeast three-hybrid strain was re-engineered so that the expression of the transcriptional activator fusion proteins and the enzyme could be regulated independently. The LexA-DHFR and B42-GR proteins were placed under the control of the fully regulatable GAL1 promoter; and the P99 cephalosporinase, under the repressible MET promoter. First, we established independently that the cephalosporinase was being expressed in an active form in the yeast cells using nitrocefin, a known chromagenic substrate for the cephalosporinase (data not shown) [37]. Then, using standard lacZ assays on X-gal plates, we developed conditions where expression of the P99 cephalosporinase disrupted Mtx-Cephem-Dex-mediated *lacZ* transcription (Fig. 5B, column 3). These results were confirmed using quantitative assays in liquid culture with ONPG (Fig. 5C) and establish that the three-hybrid assay can be used to detect cephalosporinase activity.

A number of experiments were carried out to confirm that the change in transcription of the reporter gene was in fact due to enzyme turnover. First, we showed that enzyme-dependent disruption of the transcription read-out was cephem dependent by comparing the levels of *lacZ* transcription with Mtx-Dex and Mtx-Cephem-Dex. Using standard *lacZ* transcription assays both on plates and in liquid culture, we determined the levels of *lacZ* transcription in yeast strains expressing different LexA and B42 fusion proteins, enzymes, and a *lacZ* reporter gene (Figs 5B and 5C).

In these assays, either no small molecule, Mtx-Dex with a non-cleavable linker at 1 μM concentration in the media, or Mtx-Cephem-Dex with the cleavable cephem linker at 10 μM concentration was used. As can be seen, *lacZ* transcription in the strain expressing LexA-DHFR and B42-GR is small-molecule dependent (Figs 5B, column 2, and 5C). Expression of the wild-type cephalosporinase enzyme disrupts this small-molecule induced transcription activation when the cells are grown in the presence of Mtx-Cephem-Dex, but not Mtx-Dex (Figs 5B, column 3, and 5C). Importantly, expression of the cephalosporinase enzyme has little effect on the levels of Mtx-Dex-activated *lacZ* transcription (Figs 5B, row 2, and 5C).

Another important control is to establish that disruption of *lacZ* transcription is due to turnover of the cephem linkage and not simply sequestration of the Mtx-Cephem-Dex substrate by the cephalosporinase enzyme. To address this question, we compared the activity of the wild-type cephalosporinase enzyme in this assay with that of an inactive mutant. For the inactive variant, a mutant enzyme in which alanine replaced the active-site serine nucleophile was prepared. Cephalosporinases are serine-protease-type enzymes, and Ser^{64} is the active-site serine known to be essential for turnover of the cephem substrate [33]. In contrast with the wild-type cephalosporinase, there was no detectable change in the levels of *lacZ* transcription for cells expressing the $Ser^{64}\rightarrow$Ala mutant enzyme (Figs 5B, columns 3 and 4, and 5C). The optimal difference in signal between the active and inactive enzyme was observed when the cells were grown in 0.5% galactose, 1.5% glucose and 134 mM methionine. Under these conditions, the three-hybrid fusion proteins are expressed at low levels, and the enzyme is maximally expressed. Together, these results establish that the change in transcription of the *lacZ* reporter gene is due to enzyme-catalysed turnover of the Mtx-Cephem-Dex substrate.

Finally, a *lacZ* screen was used to isolate the wild-type cephalosporinase from a pool of inactive variants (Fig. 6). A mixture of plasmids encoding the wild-type enzyme (5 %) or the $Ser^{64}\rightarrow$Ala variant (95 %) was transformed en masse into a yeast strain expressing the LexA-DHFR and B42-GR fusion proteins and bearing a *lacZ* reporter plasmid. The resulting transformants were plated on X-gal plates and screened based on their levels of β-galactosidase expression. Initially, the screen suffered from a high percentage of false positives and false negatives. Integration of the genes encoding the LexA-DHFR and B42-GR fusion proteins, however, stabilized the transcription read-out without significantly affecting the sensitivity of the assay. Using the integrated strains, approximately 5% of the cells showed reduced levels of β-galactosidase expression, as would be expected based on the plasmid ratio.

The plasmids encoding the enzyme were extracted from 5 blue and 5 white colonies and sequenced. Sequencing confirmed that 5/5 of the blue cells contained the inactive $Ser^{64}\rightarrow$Ala mutant enzyme and 4/5 of the white cells contained the wild-type cephalosporinase enzyme. A secondary *lacZ* screen with and without Mtx-Dex could rule out false positives. The *lacZ* screen demonstrates that the yeast three-hybrid system can be used reliably to screen libraries of proteins based on catalytic activity.

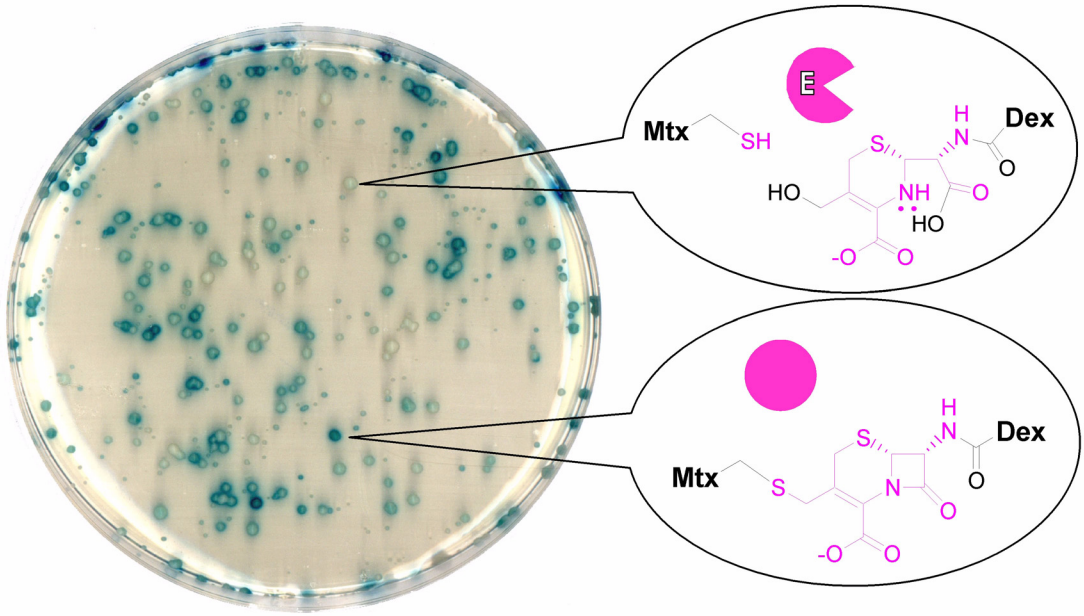


Figure 6. High-throughput Chemical Complementation Screen. Active enzyme can be isolated from a pool of inactive mutants. The yeast selection strain was transformed with a 5:95 mixture of plasmids encoding the wt active cephalosporinase enzyme and the inactive $Ser^{64}\rightarrow$Ala cephalosporinase variant, respectively, and then plated onto an X-gal indicator plate containing 10 mM Mtx-Cephem-Dex. Cells containing the active enzyme could be distinguished based on the levels of X-gal hydrolysis and hence *lacZ* transcription.

Having established that the chemical complementation system could read-out cephalosporinase activity, we next evaluated the ability of the system to distinguish enzymes based on their levels of catalytic activity [38]. A series of P99 cephalosporinase variants were designed that spanned several orders of magnitude in their catalytic activity. These variants were over-expressed and purified, and then their activity with the Mtx-Cephem-Dex substrate was determined *in vitro* using Michaelis-Menten kinetics. Next these same variants were introduced into the chemical complementation system, and their levels of transcription activation were determined using standard assays for *lacZ* transcription in liquid culture.

For all of the variants tested, the catalytic efficiency (k_{cat}/K_M) of the variant correlated with its level of *lacZ* activation in the three-hybrid assay (Fig. 7). The more active variants showed decreased levels of *lacZ* transcription, presumably because they were able to cleave the Mtx-Cephem-Dex substrate efficiently. While the less active variants showed higher levels of *lacZ* transcription. Already just using these initial conditions and a single reporter gene, the assay was able to distinguish variants spanning almost three-orders of magnitude in k_{cat}/K_M. The ability to distinguish enzymes based on their catalytic activity will be crucial for directed evolution experiments where the goal is to evolve variants with increased activity.

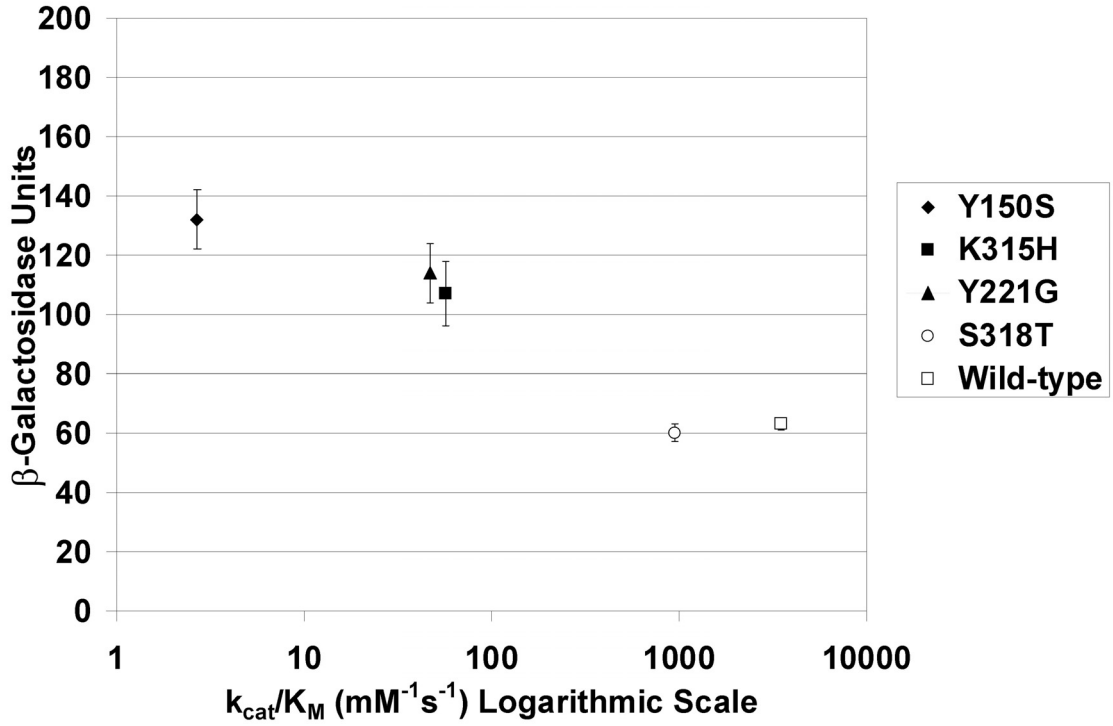


Figure 7. Correlation between catalytic efficiency and in vivo transcription of the wild-type P99 β-lactamase and its variants. Errors in k_{cat}/K_m were negligible and not plotted. Standard deviations for *lacZ* transcription activation are shown. The standard deviation for the wild-type P99 is 2 units, and is subsumed by the symbol on the graph.

Finally, in unpublished results, we have used the chemical complementation system for the directed evolution of an enzyme that catalyses the formation of a glycosidic bond (Lin, Tao and Cornish, V. unpublished results). Significantly, this result shows the generality of the approach and the ease with which it can be applied to new chemical reactions. This result also demonstrates that chemical complementation can detect bond formation as well as bond cleavage reactions and that enzyme catalysis can be linked to a growth selection as well as a *lacZ* screen.

CONCLUSION

While demonstrated with the cephalosporinase enzyme, the advantage of this selection strategy should be its generality. The yeast three-hybrid system should be able to link both bond cleavage and bond formation reactions to transcription of a reporter gene. In the case of bond cleavage reactions, the enzyme should ensure cell survival by cleaving a Mtx-Dex substrate and disrupting transcription of a toxic reporter gene. For bond formation, the enzyme should form a bond between Mtx and Dex, activating transcription of an essential reporter gene. The read-out system - Mtx-Dex, LexA-DHFR, B42-GR, and the reporter gene - can all remain constant while the chemistry changes. Thus, all that needs to be changed for each new reaction is the Mtx-SUBSTRATE-Dex or Dex-SUBSTRATE and Mtx-SUBSTRATE molecules synthesized in the lab and the enzyme library. This assay could be used to engineer glycosyltransferases, aldolases, esterases, amidases, and Diels-Alderases - all with a variety of substrate specificities and regio- and stereo-selectivities. By converting the assay to a coupled enzyme assay, it may even be possible to detect oxidases and reductases. In addition to providing a powerful selection for the evolution of enzymes with new activities, there should be many uses for a reaction-independent, high-throughput assay for enzyme catalysis. The assay can be used to study enzyme function, either to test hundreds of mutants to identify amino acids important for the catalytic activity of an enzyme or hundreds of different molecules to determine the substrate specificity of an enzyme. Likewise, the assay could be applied to drug discovery by screening libraries of small molecules based on inhibition of enzyme activity and a change in transcription of the reporter gene. A distinct advantage is that a new assay would not have to be developed for each new enzyme target. Finally, this assay should be particularly well suited to proteomics. A battery of Mtx-Dex substrates with different substrates as linkers could be prepared and then used to screen cDNA libraries to identify enzymes that fall into common families, such as glycosidases or aldolases. Since mammalian, as well as yeast, three-hybrid assays are standard, the assays could be carried out in the endogenous cell line ensuring correct post-translational modification of the proteins. The key to all of these applications is a robust assay for enzymatic activity.

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