# Correlation between Catalytic Efficiency and the Transcription Read-Out in Chemical Complementation: A General Assay for Enzyme Catalysis†

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ABSTRACT: High-throughput assays for enzyme catalysis that can be applied to a broad range of chemical reactions are key to advances in directed evolution and proteomics. Recently, we reported such a general assay, chemical complementation, which links enzyme catalysis to reporter gene transcription in vivo using the yeast three-hybrid assay. In this proof-of-principle experiment, it was shown that a wild-type $\beta$-lactamase enzyme could be isolated from a pool of inactive mutants using a *lacZ* screen. Ideally, however, such an assay should be able to distinguish enzymes based on their catalytic activity. Thus, here, we set out to determine if the catalytic efficiency of an enzyme variant does in fact correlate with its level of transcription activation in the chemical complementation assay. First, the reaction mechanism for the cleavage of the $\beta$-lactam substrate used in the chemical complementation proof-of-principle experiment was determined. Then a series of $\beta$-lactamase variants was designed to span several orders of magnitude in $k_{cat}/K_m$. The activity of each variant was determined both in vitro using purified enzyme and in vivo in the chemical complementation transcription assay. $\beta$-Lactamase variants spanning three-orders of magnitude in $k_{cat}/K_m$ could be distinguished in the assay, and the catalytic efficiency of each variant correlated with its level of transcription activation in vivo. These results establish the suitability of chemical complementation for the directed evolution of enzymes with improvements in catalytic activity and for profiling the relative substrate specificities of a group of enzymes in proteomics applications.

Directed evolution has emerged as a powerful method for improving the activity of a known enzyme and, more recently, even modifying substrate specificity (*1*−*8*). Directed evolution involves generating large pools of protein variants ($>10^4$) and then assaying these variants en masse for the desired function. While a powerful approach, directed evolution is restricted to enzymes that are inherently screenable or selectable, for example, enzymes in which the product is fluorescent or an essential metabolite. Recently, several laboratories have reported high-throughput assays for enzyme catalysis intended to be general in an effort to expand the range of enzyme activities to which directed evolution can be applied (*9*−*13*). In the approach recently developed in our laboratory, termed chemical complementation, the yeast three-hybrid assay is used to link enzyme catalysis to reporter gene transcription in vivo (Figure 1) (*14*). Proof-of-principle experiments have shown that these assays can be used to isolate the wild-type enzyme from a pool of inactive variants. However, the correlation between enzyme activity and the assay read-out really has not been explored. Thus, before we used chemical complementation for directed evolution, we first wanted to establish that there really was a correlation between enzyme activity and the transcription read-out.

In our initial report, we chose cephalosporin hydrolysis by the *Enterobacter cloacae* P99 $\beta$-lactamase (P99) as a well-studied cleavage reaction around which to develop chemical complementation. Cephalosporins are $\beta$-lactam antibiotics, and $\beta$-lactamases are the bacterial resistance enzymes that hydrolyze and inactivate these antibiotics. The P99 $\beta$-lactamase is well characterized biochemically and structurally (*15*, *16*), and the synthesis of cephalosporins is well established (*17*). The small molecule ligands were incorporated at the C3′ and C7 positions of the cephem core. Cleavage of the $\beta$-lactam bond in cephalosporins results in expulsion of the leaving group at the C3′ position, thereby effectively breaking the bond between the two small molecule ligands. We showed that chemical complementation could be used to detect $\beta$-lactamase activity using a dexamethasone-methotrexate (Dex-Mtx) heterodimer with a cephem linker (Dex-Cephem-Mtx). We also showed that the system could distinguish the wild-type P99 $\beta$-lactamase from a large pool of inactive P99:S64A variants, where the critical active-site Ser nucleophile has been mutated to an Ala.

For such an assay to be truly useful for directed evolution and proteomics, however, it must be able not only to distinguish fully active enzymes from fully inactive ones but to really distinguish enzymes based on their catalytic

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[1] Abbreviations: Dex, dexamethasone; Mtx, methotrexate; DCM, Dex-Cephem-Mtx; DBD, DNA-binding domain; AD, activation domain; DMF, dimethylformamide; DHFR, dihydrofolate reductase; GR, glucocorticoid receptor; ONPG, *o*-nitrophenyl $\beta$-D-galactopyranoside; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

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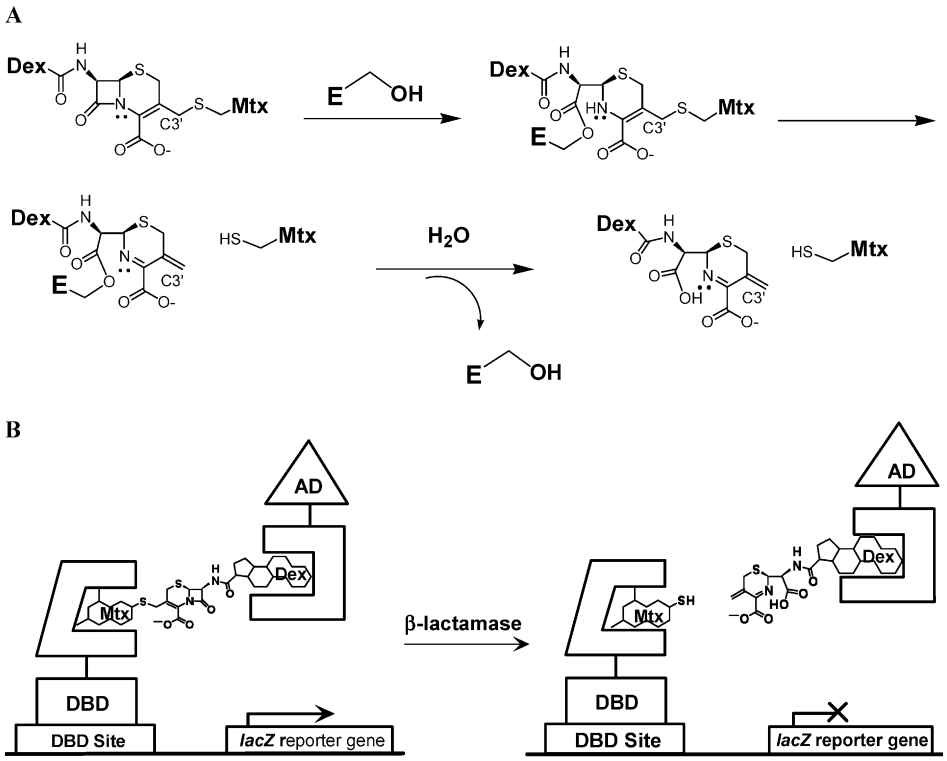



FIGURE 1: Chemical complementation. A reaction-independent complementation assay for enzyme catalysis based on the yeast three-hybrid assay. (A) $\beta$-Lactam hydrolysis provides a simple cleavage reaction around which to develop the complementation strategy. $\beta$-Lactamases are serine-protease type enzymes that catalyze the hydrolysis of $\beta$-lactam antibiotics via an acyl-enzyme intermediate. Hydrolysis of the $\beta$-lactam bond in dexamethasone-cephem-methotrexate (Dex-Cephem-Mtx) results in expulsion of the leaving group at the C3′ position of the cephem core, thus breaking the bond between Dex and Mtx. (B) Chemical complementation links enzyme catalysis to reporter gene transcription in vivo using the yeast three-hybrid assay. This assay detects enzyme catalysis of bond formation or bond cleavage reactions based on covalent coupling of two small molecule ligands in vivo. A heterodimeric ligand reconstitutes a transcriptional activator, turning on transcription of a reporter gene. Here Dex-Cephem-Mtx is used as the heterodimeric ligand. The Dex-Cephem-Mtx substrate bridges a DNA-binding domain (DBD)-receptor fusion protein and an activation domain (AD)-receptor fusion, activating transcription of the *lacZ* reporter gene. Addition of the $\beta$-lactamase enzyme results in cleavage of the Dex-Cephem-Mtx substrate and disruption of *lacZ* transcription. 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.

efficiency. Thus, before we attempted to apply chemical complementation to the directed evolution of a novel activity, we wanted to further use the $\beta$-lactamase model system to test the hypothesis that there would be a correlation between enzyme activity and the transcription read-out. Particularly with the complexity of eukaryotic transcription activation, it is not a given that such a correlation would exist. The ability to distinguish gradations in activity would add significantly to the utility of a high-throughput assay for enzyme catalysis. For directed evolution experiments, it would allow enzymes to be selected based on improvements in catalytic activity. Similarly, point mutants of enzymes with known function could be scored for their contribution to the catalytic activity of the enzyme. A series of small molecules could be screened to find the most potent inhibitor of an enzyme. And for proteomics, the substrate specificities of a group of related enzymes could be compared.

Here, we test the ability of chemical complementation to distinguish enzymes based on their catalytic activity. First, the kinetic scheme for the P99 $\beta$-lactamase with the synthetic substrate Dex-Cephem-Mtx was determined. Two independent assays were developed to measure both cleavage of the $\beta$-lactam bond and expulsion of the Mtx-thiol group. These assays allowed us to determine whether expulsion of the Mtx-thiol leaving group is the rate-determining step for Dex-Cephem-Mtx hydrolysis by the P99 $\beta$-lactamase. Then the activity of a series of P99 $\beta$-lactamase variants were determined both in vitro using purified enzyme and in vivo in the chemical complementation assay. On the basis of previous reports for $\beta$-lactam antibiotics with a similar cephem core (*15*, *18*−*26*), P99 $\beta$-lactamase variants were designed that should span several orders of magnitude in catalytic efficiency with Dex-Cephem-Mtx. These P99 variants were overexpressed and purified, and then their activities with the Dex-Cephem-Mtx substrate were determined. The activities of these same variants were also determined in the chemical complementation assay using standard *lacZ* transcription assays in liquid culture with *o*-nitrophenyl $\beta$-D-galactopyranoside (ONPG). Here we compare the catalytic efficiencies of the P99 $\beta$-lactamase variants as measured in vitro with their levels of transcription activation in the chemical complementation assay. We then discuss the implications of these results for the utility of chemical complementation as an assay for enzyme catalysis.

## EXPERIMENTAL PROCEDURES

*Materials.* Standard protocols for molecular biology and yeast genetics were used. Restriction enzymes, Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Pfu Turbo polymerase and XL1 blue cells were purchased from Stratagene (La Jolla, CA). The dNTPs used in the polymerase chain reaction

Table 1: Strains and Plasmids Used in This Study

| name | description | source/ref |
|---|---|---|
| strains | genotype | |
| BL21(DE3) | | Novagen |
| XL1 Blue | | Stratagene |
| FY250 | MATα trp1Δ63 his3Δ200 ura3−52 leu2Δ1 $GAL^+$ | D. McNabb |
| FY251 | MATa trp1Δ63 his3Δ200 ura3−52 leu2Δ1 $GAL^+$ | M. Carlson |
| V947Y | MATa ade4::$P_{gal1}$-lexA-eDHFR(HIS3) ade2:: $P_{gal1}$-B42-(GSG)2-rGR2(TRP1) ura3−52 leu2Δ1 $GAL^+$ | (*14*) |
| plasmids | details | |
| pMW112 | *8lexAop-lacZ* 2$\mu$ $kan^R$ | (*46*)/(*47*) |
| pRSS425Met | $P_{met}$ 2$\mu$ $spec^R$ LEU2 | (*14*) |
| pVC172 | $P_{met}$/P99$his_6$ 2$\mu$ $spec^R$ LEU2 | (*14*) |
| pCB687 | pVC172 with S64A mutation | (*14*) |
| pBC1149 | pVC172 with Y221G mutation | this study |
| pDS1190 | pVC172 with Y150S mutation | this study |
| pDS1191 | pVC172 with K315H mutation | this study |
| pDS1195 | pVC172 with S318T mutation | this study |
| pNU602 | P99 β-lactamase | (*18*) |
| pET26b | $P_{T7}$ $kan^R$ | Novagen |
| pSG430 | $P_{T7}$ /P99 β-lactamase-$his_6$ $kan^R$ (pET26b−P99$his_6$) | (*14*) |
| pDS984 | pSG430 with S64A mutation | this study |
| pDS1186 | pSG430 with Y221G mutation | this study |
| pDS1196 | pSG430 with Y150S mutation | this study |
| pDS1197 | pSG430 with K315H mutation | this study |
| pDS1199 | pSG430 with S318T mutation | this study |

(PCR) were purchased from Amersham Biosciences. BL21 (DE3) cells, pET26b plasmid, and the BugBuster protein extraction reagent were purchased from Novagen (Madison, WI). Cephalothin, Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid, DTNB), bovine serum albumin (BSA), and ONPG were purchased from Sigma. The transformation of *Eschericia coli* was carried out by electroporation using a Bio-Rad *E. coli* pulser. The Ni-NTA spin columns used to purify the proteins, as well as the 6× His protein ladder and Penta-His anti-His tag IgG, were purchased from Qiagen (Valencia, CA). The ECF Western blotting reagent pack, which included the anti-mouse IgG linked alkaline-phosphatase, was purchased from Amersham Pharmacia Biotech. Slide-a-Lyzer dialysis cassettes were purchased from Pierce. Oligonucleotides were purchased from the Great American Gene Company (Ramona, CA). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from American Bio-Organics. The frogger used to transfer cells was purchased from Dan-Kar Corp (Wilmington, MA). *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) and acrylamide:bis-acrylamide (37.5:1) for making 10% acrylamide/bis-acrylamide gels were purchased from Fisher (Pittsburgh, PA). Methotrexate was a gift from the National Cancer Institute (NCI). All other chemicals were purchased from Aldrich. All solutions were made with distilled water prepared from a Milli Q Water Purification system. For PCR, a MJ Research PTC-200 Peltier Thermal cycler was employed. Ultraviolet−visible measurements were taken using a Molecular Devices Spectramax 384. Sequencing was performed by GeneWiz Inc. (North Brunswick, NJ). Plasmid pNU602 encoding the *E. cloacae* P99 class C β-lactamase was kindly provided by J. Frere. The P99 gene on this plasmid contained five point mutations: I16V, A88P, and A299V, and two silent mutations $Thr^{42}$ ACA to ACG and $Ala^{292}$ GCA to GCG. The vector pRS425Met was provided by H. Madhani, the *Saccharamyces cerevisae* strain FY250 was provided by D. McNabb, and the *S. cerevisae* strain FY251 was provided by M. Carlson.

*Small Molecule*. The synthesis of dexamethasone-cephem-methotrexate (Dex-Cephem-Mtx) (Figure 2) has been described previously (*14*) and was modified to improve the yield as well as eliminate $\Delta^2$ isomerization of the cephem core (H. Lin, V. Cornish, unpublished results). The small molecule was dissolved in *N*,*N*-dimethylformamide (DMF), and the exact concentrations were determined by Beer's law, using an extinction coefficient at 385 nm of $\epsilon = 5600$ $cm^{-1}$ $M^{-1}$ at 27 °C in DMF (determined from a solution of methotrexate of known concentration in DMF). The solutions were stored at −80 °C. A spectral scan was taken before and after complete hydrolysis of the Dex-Cephem-Mtx with 1 μM P99 β-lactamase. The maximal change in absorbance upon hydrolytic cleavage of the β-lactam bond in the cephem core was found to be at 265 nm. $\Delta\epsilon$ for the small molecule was determined by plotting the change in absorbance after completion of hydrolysis with 1 μM P99 at the substrate concentrations 10, 20, 30, 40, and 50 μM. Dex-Cephem-Mtx was found to have a $\Delta\epsilon_{265}$ of 7600 $M^{-1}$ $cm^{-1}$. For comparison, cephalothin has a $\Delta\epsilon_{265}$ of 7700 $M^{-1}$ $cm^{-1}$ (*27*).

*Plasmid Construction*. Plasmids used in this study are listed in Table 1. For expression in *S. cerevisiae*, plasmid pRSS425Met encoding the *E. cloacae* P99 class C β-lactamase mutant lacking the N-terminal periplasmic leader sequence and containing a C-terminal $histidine_6$ ($His_6$) tag was used (*14*). The P99 β-lactamase mutant Y221G was created by QuikChange site-directed mutagenesis according to the manufacturer's protocol using the pRSS425Met-P99$his_6$ plasmid (*14*) and the following primers for mutagenesis: VC554 5′-CTG TTC GAG CTG GGT GCT ATA AGT AAA ACC TTC and VC555 5′-GAA GGT TTT ACT TAT AGC ACC CAG CTC GAA CAG to generate plasmid pCB687 (S64A); VC1183 5′- G CTG GAT GCA CAA GCC GGC GGC GTG AAA ACC AAC G and VC1184 5′- C GTT GGT TTT CAC GCC GCC GGC TTG TGC ATC CAG C to generate plasmid pBC1149 (Y221G). The genes encoding the *E. cloacae* P99 class C β-lactamase mutants Y150S, K315H, and S318T were amplified from plasmids

encoding the P99 mutants constructed in another study (*23*). A 1107-bp Pst I to Hind III fragment encoding the *E. cloacae* P99 mutant of interest was created by using primers VC28 5′-GCA TAC GTC CTG CAG ATG ACG CCA GTG TCA GAA AAA C (Pst I, coding strand) and VC69 5′-GCA TTG CTG AAG CTT AGT GGT GGT GGT GGT GGT GCT GTA GCG CCT CGA GG (Hind III, noncoding strand). This fragment was inserted between the Pst I and Hind III sites in pRSS425Met to generate the plasmids pDS1190, pDS1191, and pDS1195. The presence of the desired mutation was confirmed by fully sequencing the enzyme coding region using the primers VC32 5′-TGG CAG GGT ATT CGT ATG (coding strand), VC33 5′-GTC ATG GCA AAC ATG GCG (coding strand), VC34 5′-GCG CAC GAG GGA GGC GTT (noncoding strand), and VC649 5′-GGC AGC CGC AGT GGA AGC C (coding strand).

For overexpression in *E. coli*, plasmid pSG430 encoding the P99 class C $\beta$-lactamase lacking the N-terminal periplasmic leader sequence and containing a C-terminal $His_6$ tag was used (*14*). The P99 $\beta$-lactamase mutants in the overexpression vector were generated using Strategene's QuikChange site-directed mutagenesis kit using double-stranded pET26b-P99$his_6$ and the following primers for mutagenesis: VC554 5′-CTG TTC GAG CTG GGT GCT ATA AGT AAA ACC TTC and VC555 5′-GAA GGT TTT ACT TAT AGC ACC CAG CTC GAA CAG to generate plasmid pDS984 (S64A); VC1183 and VC1184 to generate plasmid pDS1186 (Y221G); VC893 5′-GGC ACA ACG CGT CTT TCT GCC AAC GCC AGC ATC and VC894 5′-GAT GCT GGC GTT GGC AGA AAG ACG CGT TGT GCC to generate pDS1196 (Y150S); VC887 5′-GCG TCC TGG GTC CAT CAC ACG GGC TCT ACT GGC GGG and VC888 5′-CCC GCC AGT AGA GCC CGT GTG ATG GAC CCA GGA CGC to generate plasmid pDS1197 (K315H); VC1293 5′-GG GTC CAT AAA ACG GGT ACC ACT GGC GGG TTT GGC AGC and VC1294 5′-GCT GCC AAA CCC GCC AGT GGT ACC CGT TTT ATG GAC CC to generate plasmid pDS1199 (S318T). The presence of only the desired mutation in plasmids pDS984, pDS1186, pDS1196, pDS1197, and pDS1199 was confirmed by fully sequencing the enzyme coding region.

*Strain Construction*. The yeast strains described in this paper are listed in Table 1. Strain V947Y was prepared by integrating the genes encoding LexA-DHFR and B42-GR under the control of the GAL1 promoter at the chromosomal loci *ade4* and *ade2* respectively in *S. cerevisiae* strain FY251 (MATa). The *S. cerevisiae* strain FY250 (MAT$\alpha$) was transformed using lithium acetate with the plasmid pMW112, which encodes the *lacZ* gene under the control of the eight tandem LexA operators and either the plasmid pRSS425Met, pVC172, pCB687, pDS1190, pDS1191, or pDS1195, and followed by selection on synthetic complete (SC) media containing 2.0% glucose and lacking the appropriate selective nutrients as described (*14*). Six colonies from each transformation of FY250 were mated with strain V947Y in Falcon round-bottom microtiter plates (Fisher) containing YPD (yeast extract/peptone/dextrose) medium for 12 h and then diluted 100× into SC medium containing 2.0% glucose and lacking histidine, tryptophan, uracil, and leucine to select for mated diploid strains.

*Liquid LacZ Transcription Assays*. Yeast strains expressing the chemical complementation system and different $\beta$-lactamase variants were assayed for $\beta$-galactosidase activity using ONPG as a substrate. Each yeast strain was initially frogged from glycerol stocks containing the mated yeast strains into synthetic complete medium lacking histidine, uracil, tryptophan, and leucine, and containing 2.0% glucose. After 3 days, 5 $\mu$L of the grown yeast strains was inoculated into 95 $\mu$L of synthetic complete medium lacking histidine, uracil, tryptophan, and leucine, and containing 0.5% galactose, 1.5% glucose, and 2.0% raffinose to turn on transcription of LexA-DHFR and B42-GR and a final concentration of either no small molecule or 5 $\mu$M Dex-Cephem-Mtx and grown for 3 days at 30 °C. The growth media was then discarded, and the yeast cells were washed in 100 $\mu$L of sterile DI water and then resuspended in 100 $\mu$L of the growth media for a optical density reading at $A_{600}$. The cultures were lysed with 1.0% SDS/chloroform in 100 $\mu$L of sterile DI water and then assayed for $\beta$-galactosidase activity using a standard liquid ONPG assay protocol (*28*) in 96-well plates. The equation to calculate $\beta$-galactosidase units was as follows, $\beta$-galactosidase units $= 1000(A_{420}/[A_{600}$*time in minutes*volume assayed in mL]). The liquid *lacZ* transcription assay was performed in duplicate.

*P99 $\beta$-Lactamase Purification*. P99 $\beta$-lactamase engineered to have a C-terminal $His_6$ tag was purified from strain BL21(DE3) carrying plasmid pSG430. A cell pellet from 500 $\mu$L of culture known to express protein based on analysis by SDS−PAGE was resuspended in 500 $\mu$L of LB media. A 1:500 dilution of this suspension was then used to inoculate 50 mL of LB containing 30 $\mu$g/mL kanamycin. This culture was grown at 37 °C with vigorous shaking to an $OD_{600}$ of 0.6−0.8, at which time expression of the enzyme was induced by adding IPTG to a final concentration of 0.5 mM. After growth for an additional 3 h, the cells were harvested by centrifugation and stored at −80 °C. The pellet was thawed at room temperature, and the cells were lysed by adding 2 mL of BugBuster protein extraction reagent and following the protocol supplied by the manufacturer. The protein was then purified under standard nondenaturing conditions using a Ni-NTA Spin kit according to the manufacturer's protocol (Qiagen). Fractions containing protein were dialyzed against PBS (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $NaH_2PO_4$, 1.4 mM $KH_2PO_4$), pH 7.0. The enzyme concentration was determined based on the $A_{280}$ assuming an $\epsilon = 71\ 000\ M^{-1}\ cm^{-1}$ as reported (*29*). The purified protein was stored at 4 °C. An identical procedure was followed for purification of the $\beta$-lactamase mutants (with cells carrying the appropriate plasmids). Yields of 500−1000 $\mu$g of protein from 50 mL of culture were obtained. The proteins were judged to be >95% pure based on Coomassie staining of a sodium dodecyl sulfate (SDS)−polyacrimide gel electrophoresis (PAGE).

*Enzyme Kinetics.* $\beta$-Lactamase activity with Dex-Cephem-Mtx was detected by a decrease in $A_{265}$. Activity was assayed in a solution composed of a 1:1 mixture of enzyme in phosphate buffered saline (PBS) and Dex-Cephem-Mtx in 10 mM sodium phosphate buffer. The final volume of the reaction was 200 $\mu$L, and the pH of both solutions was 7.0. Reactions were started with the addition of 250 pM wild-type P99 $\beta$-lactamase (100 $\mu$L of a 500 pM solution in assay buffer) to 100 $\mu$L of 2× the final concentration of Dex-Cephem-Mtx (1−100 $\mu$M final concentration from a 8.35

mM stock solution in DMF) solution in wells, and the progress was monitored by UV. Kinetic constants were determined by initial velocity nonlinear regression analysis. The initial velocities were measured for eight substrate concentrations, and then fit to the Michaelis−Menten equation, $v_0 = V_{max}[S]/(K_m + [S])$ using Kaleidagraph (Synergy Software, Reading, PA). All reactions were carried out at 27 °C. The values for the $\beta$-lactamase mutants were performed as described with the exception that the final concentration of enzyme in the assay was 1 nM for the S318T mutant, 10 nM for the K315H and Y221G mutants, and 100 nM for the Y150S mutant. Kinetic constants were attempted over enzyme concentrations, 100 nM to 2 $\mu$M, for the S64A mutant, but we were not able to detect any measurable activity with this mutant.

The rate of expulsion of the Mtx-thiol group from the C3′ position of Dex-Cephem-Mtx was detected by monitoring the reduction of Ellman's reagent. The rate constant was determined by performing the reaction in a stoppered cuvette in a total volume of 700 $\mu$L using 10 nM wild-type P99 $\beta$-lactamase, 25 $\mu$M DCM (25 $\mu$M Dex-Cephem-Mtx final concentration from a 8.35 mM stock solution in dimethylformamide), and 50 $\mu$M DTNB (50 $\mu$M final concentration from a solution in a 10 mM 10% DMF/10 mM sodium phosphate buffer, pH 7.0) for 12 h at 27 °C. The reaction was started by adding 350 $\mu$L of 20 nM P99 $\beta$-lactamase to a cuvette containing 125 $\mu$L of both 4× concentrated Dex-Cephem-Mtx (100 $\mu$M) and DTNB (200 $\mu$M) in sodium phosphate buffer, pH 7.0. The reaction was blanked with an equivalent concentration of Dex-Cephem-Mtx and DTNB to correct for the breakdown of Dex-Cephem-Mtx without the presence of enzyme. The data were then fit to a first-order single exponential to determine the value of $k_4$. Subsequent rates were assayed in a solution of 200 $\mu$L in microtiter plates containing 50 $\mu$L of 100 $\mu$M Dex-Cephem-Mtx (25 $\mu$M final concentration) and 50 $\mu$L of 200 $\mu$M DTNB (50 $\mu$M final concentration) in sodium phosphate buffer, pH 7.0. Reactions were performed at 27 °C and started with the addition of either 1, 10, or 100 nM P99 $\beta$-lactamase (100 $\mu$L of a 2, 20, or 200 nM solution in PBS). The reactions were referenced with an equivalent amount of Dex-Cephem-Mtx and DTNB. DTNB was maintained in a 2-fold excess of the Dex-Cephem-Mtx substrate in all reaction assays. All reactions were carried out in duplicate. The data were collected over 2 h and fit to a first-order single exponential to determine the rate constant $k_4$. The values for the $\beta$-lactamase mutants were performed as described in microtiter plates with the exception that the final concentration of enzyme in the assay was 2 $\mu$M for the P99:S318T, P99:K315H, P99:Y221G, and P99:Y150S mutants.

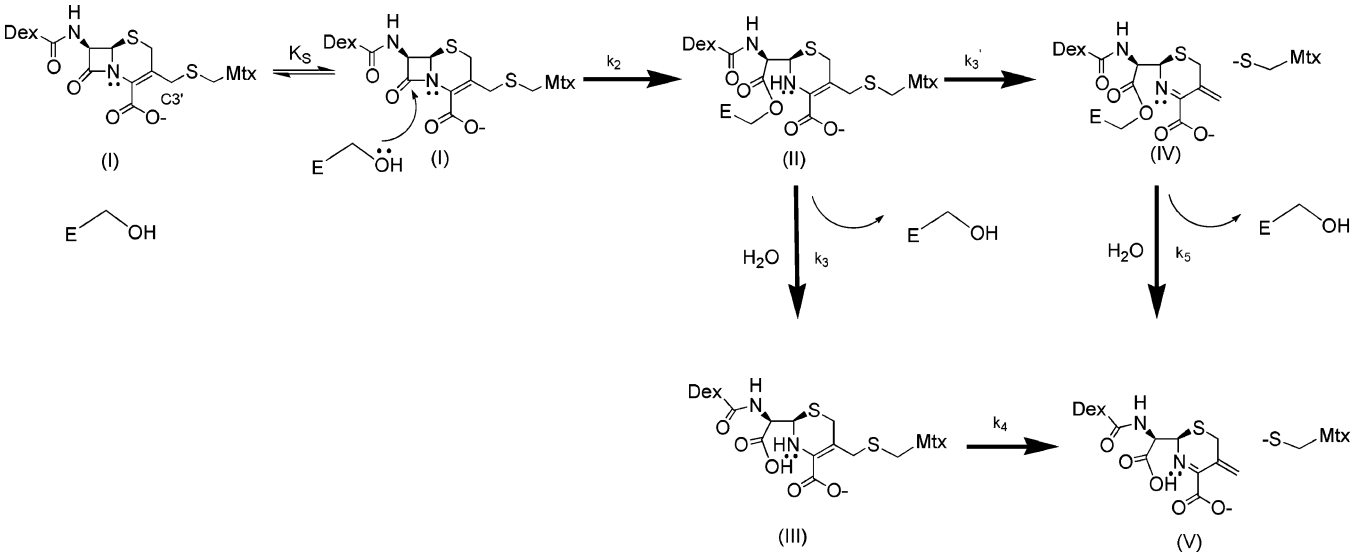
To confirm that the presence of excess DTNB did not alter the activity of the $\beta$-lactamases (*30*), the concentration of DTNB was varied between 1-, 5-, and 10-fold excess over the concentration of Dex-Cephem-Mtx in solution. The reaction was started by the addition of 10 nM P99 (100 $\mu$L of a 20 nM solution in assay buffer) to 50 $\mu$L of 200 $\mu$M cephalothin (100 $\mu$M final concentration) and 50 $\mu$L of 4× concentrated DTNB (final concentrations 50 $\mu$M to 1 mM from a 10 mM stock solution of 10% DMF/10 mM sodium phosphate buffer pH 7.0). Since both Dex-Cephem-Mtx and DTNB need to be solubilized in dimethylformamide, the concentration of DMF was varied between 1, 5, and 10% of the total volume of the reaction (200 $\mu$L performed in a plate) and the effects on the rate of hydrolysis of 100 $\mu$M cephalothin were monitored. The reaction was started by the addition of 10 nM P99 (100 $\mu$L of a 20 nM solution in assay buffer) to wells containing 100 $\mu$L of 200 $\mu$M cephalothin. The hydrolysis of cephalothin was monitored at 265 nm by UV.

*Protein Expression Levels*. Western blots were carried out to assess the protein expression levels of the wild-type P99 $\beta$-lactamase and its variants. Yeast three-hybrid strains expressing the different $\beta$-lactamase variants were grown to mid-log phase ($OD_{600} = 0.5-0.9$) in 10 mL of the appropriate SC media containing 0.5% galactose/1.5% glucose/2.0% raffinose and then lysed using acid-washed glass beads. Total protein concentrations were determined by Bradford assays (Bio-Rad, CA) with bovine serum albumin (BSA) as a standard. Relative protein expression levels were determined by normalization such that 1 $\mu$g of total protein of each variant and 4 $\mu$L of the $His_6$ ladder was loaded onto a SDS−PAGE, followed by transfer to a PVDF membrane and Western staining. The immunodetection was followed according to the protocol provided in the QIA Express Detection and Assay Handbook (Qiagen). The PVDF membrane was stained with the primary antibody at a 1:2000 dilution (Penta-His anti-His tag IgG), followed by incubation with the secondary antibody (anti-mouse IgG linked to alkaline phosphatase) at a 1:2500 dilution according to standard protocols. The secondary antibody was detected by the incubation of the PVDF membrane with 1 mL of the fluorescent substrate ECF in solution (prepared according to Amersham Pharmacia Biotech's protocol) for 10 min. The membrane was visualized by fluorescence scanning using the Storm PhosphorImager (Amersham). The proteins were quantitated by loading 1 $\mu$g of total wild-type P99 protein extract and comparing the pixel density of this band to the pixel densities in the 50 ng to 2 $\mu$g of the purified P99 $\beta$-lactamase bands. The bands were analyzed by the ImageQuant program.

## RESULTS

To determine if chemical complementation could in fact distinguish enzymes based on their catalytic activity, we sought to compare the levels of transcription activation for a series of P99 $\beta$-lactamase variants in the chemical complementation assay to their catalytic efficiencies in vitro. Both of the small molecule ligands Dex and Mtx are buried deeply in their respective receptor cores, and the P99 $\beta$-lactamase encapsulates the cephem core. Further, the $\beta$-lactamase has been localized to the cytoplasm, while the transcription activation components have been localized to the nucleus. Thus, we assume that the P99 $\beta$-lactamase can only act on Dex-Cephem-Mtx unbound by the transcriptional activator fusion proteins. The in vitro activity of the P99 $\beta$-lactamase with free Dex-Cephem-Mtx, as opposed to the ternary complex, is the appropriate model. Since the activity of $\beta$-lactamases and $\beta$-lactamase variants, of course, is substrate dependent, the catalytic efficiency of the variants needed to be determined explicitly for the Dex-Cephem-Mtx substrate. The first step then was to determine the kinetic scheme for the P99 $\beta$-lactamase with Dex-Cephem-Mtx.

Scheme 1 : Complete Hydrolysis and Enzymatic Turnover of a Cephalosporin Antibiotic



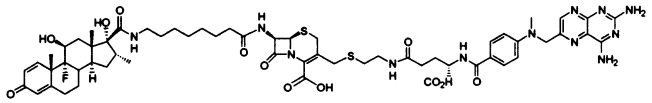



FIGURE 2: Structure of the Dex-Cephem-Mtx substrate.

*Determination of the Kinetic Scheme for the P99 β-Lactamase with Dex-Cephem-Mtx.* As has been described by Pratt, the relative rates of hydrolysis of the acyl-enzyme intermediate and expulsion of the leaving group at the C3′ position of the cephem core (here, Mtx) are highly substrate and enzyme-dependent. It is well documented that the complete turnover of the β-lactamase can occur via two different pathways (*31*−*35*). As outlined in Scheme 1, elimination can occur through a pathway where direct hydrolysis of the acyl-enzyme intermediate (II) completes enzymatic turnover (III) prior to the expulsion of the substituent at C3′. Alternatively, expulsion of the leaving group from (II) can first lead to a second, more inert acyl-enzyme intermediate (IV), which then undergoes hydrolysis also completing enzymatic turnover. The β-lactamase can be regenerated following the hydrolysis of the β-lactam bond via (III) or after the hydrolysis of the bond and expulsion of the leaving group (IV). For first generation cephalosporins with good leaving groups such as cephaloridine and cephalothin expulsion of the leaving group is the rate-determining step for both the P99 class C β-lactamase and the TEM-2 class A β-lactamase (*34*, *35*). Whereas for cephamycins and third-generation cephalosporins, the rapid elimination of the leaving group gives rise to a better antibiotic due to the formation of a more inert acyl-enzyme intermediate. The reactivity of any given substrate by a β-lactamase, however, is complex and not simple to predict. For example, hydrolysis of cephalothin by the PC1 class A β-lactamase results in the leaving group being expelled prior to deacylation (*31*). The pathway through which the turnover of (II) leads to the final product (V) is dependent not only on the nature of the substrate, but also on the β-lactamase catalyst. Thus, we first set out to determine the reaction pathway of the P99 β-lactamase with our synthetic Dex-Cephem-Mtx substrate in vitro. Two separate assays were developed to measure cleavage of the β-lactam bond and expulsion of the Mtx-thiol group independently.

The P99 β-lactamase was overexpressed in *E. coli* from a T7 promoter and purified by nickel-affinity chromatography using a $His_6$ tag. The protein was judged to be >95% pure based on SDS−PAGE and staining with Coomassie. The Dex-Cephem-Mtx substrate (*14*) was synthesized by an optimized protocol that eliminates $\Delta^2$ isomerization and will be reported in a separate publication (H. Lin, V. Cornish, unpublished results). The structure of Dex-Cephem-Mtx (Figure 2) was confirmed by both 1D (see Supporting Information for 1D NMR) and 2D $^1$H NMR spectroscopy and mass spectrometry.

Steady-state kinetic constants for the reaction of the purified proteins with Dex-Cephem-Mtx were determined by monitoring the change in UV absorbance at 265 nm upon cleavage of the β-lactam bond. The rate of cleavage was shown to be enzyme dependent. Initial velocities were determined at eight different substrate concentrations, from 1−100 μM for Dex-Cephem-Mtx, and these data were fit to the Michaelis−Menten equation by nonlinear regression analysis. (See Supporting Information for fits.) The $k_{cat}$ value was determined to be 190 (±10) $s^{-1}$ for the wild-type P99 β-lactamase, while the value of $K_m$ was found to be 54 (±8) μM, which results in a calculated $k_{cat}/K_m$ of $3.5 \times 10^3$ $mM^{-1}$ $s^{-1}$. The highest substrate concentration that could be used was 100 μM due to the absorbance from methotrexate in the region of 265 nm. The $k_{cat}/K_m$ could not be determined directly, as the change in UV absorbance was too small at low substrate concentrations. These values are similar to those reported for cephalothin with the P99 β-lactamase (*23*), $k_{cat} = 153$ $s^{-1}$ and $K_m = 5.7$ μM. The Michaelis−Menten parameters are that for a typical serine protease-type enzyme. The $k_{cat}$ then is $k_2k_3/(k_2 + k_3)$. Assuming $k_2 \gg k_3$, which is a reasonable assumption for the P99 β-lactamase (*36*), then $k_{cat} \sim k_3$. The UV assay, therefore, allows determination of $k_3$.

A coupled assay using Ellman's reagent allows for the independent measurement of the rate of expulsion of the methotrexate thiol derivative (Mtx-SH) from the C3′ position of the small molecule and the calculation of the rate constant

Scheme 2 : Hydrolysis and Cleavage of Dex-Cephem-Mtx by the P99 $\beta$-Lactamase

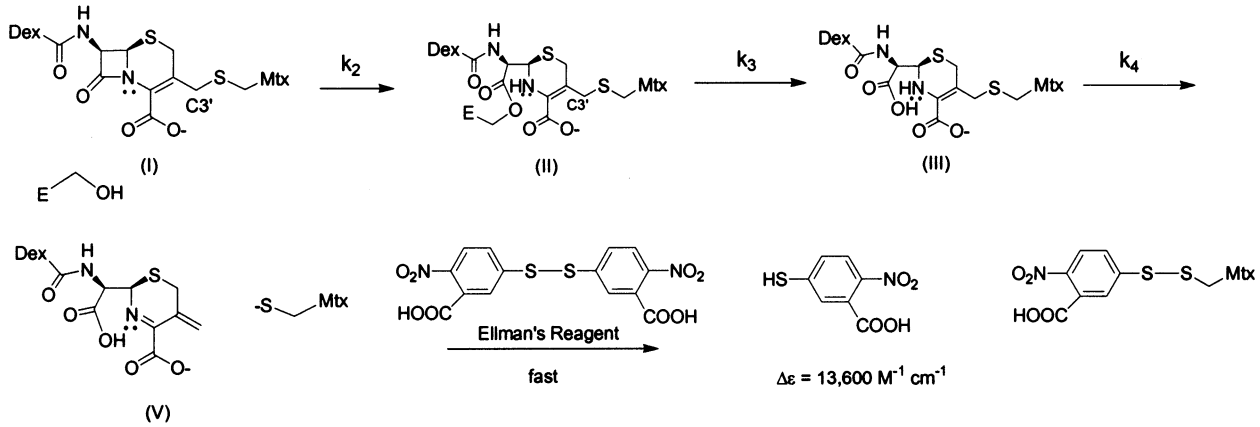



Table 2: Rate of Expulsion of Mtx-Thiol by the Wild-Type P99 $\beta$-Lactamase[a]

| wt P99 (nM) | $k_4$ ($s^{-1}$) |
|---|---|
| 1 | $4.1 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) |
| 10 | $4.8 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) |
| 100 | $4.7 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) |

[a] The rate of expulsion of the leaving group from the C3′ position of Dex-Cephem-Mtx was determined by the addition of enzyme to a reaction mixture containing the small molecule and the reporter substrate, DTNB, in 10 mM sodium phosphate buffer, pH 7.0, at room temperature. Mtx-thiol that was released into the solution was instantaneously reduced by DTNB, and the product ($\Delta\epsilon = 13\,600\ M^{-1}\ cm^{-1}$) was monitored at 412 nm.

$k_4$. Expulsion of the Mtx-SH group gave an increase in absorbance at 412 nm due to the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, $\epsilon_{412} = 13\,600\ M^{-1}\ cm^{-1}$) as shown in Scheme 2. The cleavage of the $\beta$-lactam bond by the P99 $\beta$-lactamase results in the formation of III. The small molecule dimerizer is cleaved after the expulsion of the Mtx-thiol, which can then be detected with the Ellman's reagent thiol trap. The cleavage results in the formation of Dex-Cephem and Mtx-thiol (V). Once it was determined that the concentration of DTNB and DMF in the reaction did not affect the activity of the enzyme, the value of $k_4$ for 25 $\mu$M Dex-Cephem-Mtx with 10 nM P99 was determined to be $4.4 \times 10^{-5}\ s^{-1}$ ($\pm 0.1 \times 10^{-5}$) by a first-order single-exponential fit for a 12 h reaction (Supporting Information). Decreasing and increasing the concentration of P99 10-fold resulted in similar values for $k_4$ (Table 2), where the rate of Mtx-SH formation was found to be $4.1 \times 10^{-5}\ s^{-1}$ ($\pm 0.1 \times 10^{-5}$) with 1 nM P99 and $4.7 \times 10^{-5}\ s^{-1}$ ($\pm 0.1 \times 10^{-5}$) with 100 nM P99. The lack of observation of a burst of product with the addition of enzyme to a reaction mixture containing Dex-Cephem-Mtx and excess DTNB further proved that the expulsion of the leaving group is enzyme independent (via II, Scheme 1).

Taken together, these results are consistent with a mechanism for the P99 $\beta$-lactamase with Dex-Cephem-Mtx where acyl-enzyme hydrolysis precedes expulsion of the Mtx-SH C3′ leaving group (Scheme 1, pathway via III). The rate constant $k_3$ is several orders of magnitude greater than the rate constant $k_4$. The expulsion of Mtx-thiol is enzyme independent. At high enzyme concentrations, varying the enzyme concentration does not affect the rate of Mtx-SH expulsion. As will be elaborated in the discussion, it is important to note that while the final step then is enzyme independent, the overall rate of Mtx-SH product formation is of course enzyme dependent.

*Design of P99 Variants.* To determine whether chemical complementation could in fact distinguish enzymes based on their catalytic efficiency, we designed a series of P99 variants expected to have different $k_{cat}/K_m$'s with Dex-Cephem-Mtx. On the basis of the activity of mutants of the P99 $\beta$-lactamase with a structurally similar cephalosporin, cephalothin, P99 variants were chosen that should span 3 orders of magnitude in $k_{cat}/K_m$ (*19*, *21*, *23*−*26*). Cephalothin possesses an identical cephem core as Dex-Cephem-Mtx, and differs at the C7 position where it bears a thiophene group and at the C3′ position where there is an acetoxy substituent. The point mutants S318T, K315H, Y221G, Y150S, and S64A were each introduced separately into the P99 $\beta$-lactamase for overexpression and purification of the P99 $\beta$-lactamase from *E. coli*, and in *S. cerevisiae* for the chemical complementation system using standard techniques. For all mutants, the P99 coding region was sequenced in full. The activities of the P99 variants were then determined in vitro and in vivo.

*In Vitro Characterization of the P99 $\beta$-Lactamase Variants*. The values for $k_{cat}$, $K_m$, and $k_4$ for the P99:S318T, P99:K315H, P99:Y221G, and P99:Y150S variants were determined as done for the wild-type P99 $\beta$-lactamase with Dex-Cephem-Mtx. While the $K_m$ values are all within an order of magnitude of the wild-type enzyme, the $k_{cat}$ values vary over a large range. The kinetic constants are summarized in Table 3. The catalytic efficiency ($k_{cat}/K_m$) of P99:S318T is an order of magnitude slower than that of the wild-type enzyme, while the P99:K315H and P99:Y221G variants are down 2 orders of magnitude; the P99:Y150S variant, by 3 orders of magnitude; and there was no measurable activity for the P99:S64A variant. The value of $k_4$, the rate constant for expulsion of Mtx-thiol, was very similar in value for all of the P99 variants to the wild-type enzyme. Again, expulsion of Mtx-thiol was rate-determining and enzyme independent. In conjunction with the Michaelis−Menten data, this result confirms that expulsion of the leaving group is enzyme independent. All of the P99 variants tested here appear to follow the same reaction mechanism as the wild-type enzyme.

*In Vivo Complementation Assay with Wild-Type P99 and P99 Variants*. Yeast strains containing the DBD-DHFR and AD-GR fusion proteins, the *lacZ* reporter gene, and either

Table 3: Kinetic Parameters for the Hydrolysis and Cleavage of Dex-Cephem-Mtx by Wild-Type P99 and P99 Variants[a]

| | WT P99 | S318T | Y221G | K315H | Y150S |
|---|---|---|---|---|---|
| $k_{cat}$ ($s^{-1}$) | 190 (±10) | 41 (±3) | 4.0 (±0.6) | 4.2 (±0.4) | 0.08(±0.01) |
| $K_m$ ($\mu$M) | 54 (±8) | 43 (±8) | 70 (±20) | 90 (±10) | 30 (±10) |
| $k_{cat}/K_m$ ($mM^{-1}\,s^{-1}$) | $3.5 \times 10^3$ | $9.5 \times 10^2$ | $5.7 \times 10^1$ | $4.7 \times 10^1$ | $2.7 \times 10^0$ |
| $k_4$ ($s^{-1}$) | $4.8 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) | $4.2 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) | $4.0 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) | $3.3 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) | $4.9 \times 10^{-5}$ ($\pm 0.1 \times 10^{-5}$) |

[a] $\beta$-Lactamase activity with the small molecule dimerizer, Dex-Cephem-Mtx, was measured for the wild-type and mutant $\beta$-lactamase variants in sodium phosphate buffer pH 7.0 at room temperature. Steady-state kinetic parameters were determined by fitting initial rates at different substrate concentrations to the Michaelis−Menten equation to obtain $k_{cat}$ and $K_m$. The rate of expulsion of the leaving group from the C3′ position of Dex-Cephem-Mtx was determined as described in the footnote in Table 2.

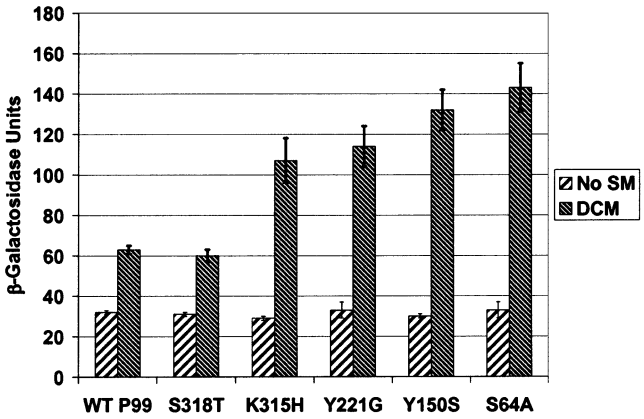



FIGURE 3: Transcription activation by the P99 $\beta$-lactamase variants in the chemical complementation assay. Yeast strains expressing the DBD−DHFR and AD−GR fusion proteins, containing the *lac*Z reporter gene, and expressing either the wild-type P99 $\beta$-lactamase or a P99 variant were grown in liquid culture and assayed for *lac*Z transcription using ONPG. The liquid culture contained either no Dex-Cephem-Mtx (No SM − no small molecule) or 5 $\mu$M Dex-Cephem-Mtx (DCM). The assay was performed for six independent colonies for each P99 $\beta$-lactamase variant. ONPG hydrolysis rates are reported as $\beta$-galactosidase units, and the error bars correspond to the standard deviation from the mean. These results show that the catalytic activity of the P99 variant correlates with its level of transcription activation in the chemical complementation assay. The more active the P99 variant, the more efficiently it cleaves the Dex-Cephem-Mtx dimerizer, and thus decreases the levels of transcription activation.

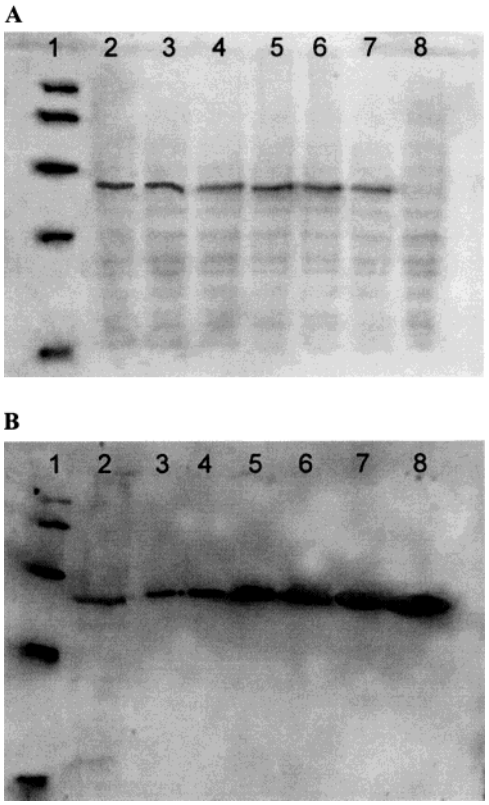



FIGURE 4: Western blot analysis of enzyme expression level. There are no significant variations in the expression levels of the wild-type P99 $\beta$-lactamase or its variants in vivo. The $His_6$ protein ladder (15, 30, 50, 75, and 100 kDa) was run in lane 1 of each gel. Lanes 2−8 in gel A correspond to yeast strains grown in 0.5% galactose/1.0% glucose/2.0% raffinose containing: 2, wild-type P99; 3, P99:S318T; 4, P99:K315H; 5, P99:Y221G; 6, P99:Y150S; 7, P99:S64A; 8, pRSS425Met with no enzyme. The enzymes show the expected molecular mass of 39 kDa (*29*). Lane 2 in gel B corresponds to 1 $\mu$g of total wild-type P99 $\beta$-lactamase yeast lysate, while lanes 3−8 contain 50, 100, 250, 500, 750, and 1000 ng of the purified wild-type P99 $\beta$-lactamase, respectively.

the wild-type or mutant P99 $\beta$-lactamases, were assayed for *lacZ* transcription in liquid culture using ONPG as a substrate under standard conditions. The levels of transcription activation were determined both with and without 10 $\mu$M Dex-Cephem-Mtx in the growth media. The levels of transcription activation with the five variants spanned the full dynamic range of the *lacZ* assay as seen in Figure 3. The wild-type P99 $\beta$-lactamase and the P99:S318T variant both showed low levels of transcription activation, as would be expected if they efficiently cleave the cephem bond in Dex-Cephem-Mtx. They both showed levels of transcription activation only 2-fold that of background transcription with no Dex-Cephem-Mtx. The P99:S64A and P99:Y150S variants both showed high levels of transcription activation as would be expected since these variants are poor catalysts of the hydrolysis of Dex-Cephem-Mtx. The P99:K315H and P99:Y221G variants both resulted in an intermediate level of transcription activation as would be expected since their activity is between that of the P99:S318T and P99:Y150S variants.

*Protein Expression Levels*. To ensure that the difference in transcription read-out did not arise from a difference in enzyme expression levels, a Western blot was carried out. Briefly, an equivalent amount of total protein was separated by SDS−PAGE, and the protein of interest was detected by an anti-His antibody. As shown in Figure 4, the expression levels of the various $\beta$-lactamases are very similar to one another. Comparison to purified P99 $\beta$-lactamase of a known concentration from $A_{280}$ allows for an estimation of the in vivo concentration of the P99 $\beta$-lactamase. Approximately 20 ng of protein was obtained from a 10-mL culture at $OD_{600} = 1$. Assuming 1 $OD_{600} \sim 10^7$ cells/mL and assuming the volume of a yeast cell is $5 \times 10^{-7}$ $\mu$L, the concentration of the $\beta$-lactamase is ~10 nM.

## DISCUSSION

Before we could evaluate the ability of chemical complementation to distinguish enzyme variants based on their catalytic efficiency, the reaction pathway of the P99 $\beta$-lactamase with Dex-Cephem-Mtx had to be determined. As Pratt has shown, either hydrolysis of the acyl-enzyme intermediate or expulsion of the leaving group at the C3′ position can be the rate-determining step, depending on both the enzyme and the substrate. Chemical complementation reads out cleavage of the bond between Dex and Mtx—here, expulsion of the Mtx-thiol from the C3′ position. Thus, it is critical to understand which step is rate-determining with Dex-Cephem-Mtx to understand the activity of P99 variants in the chemical complementation assay. Kinetic studies using two independent assays to measure cleavage of the $\beta$-lactam bond and expulsion of Mtx-thiol support a mechanism in which the latter is rate-determining and enzyme independent for Dex-Cephem-Mtx with the P99 $\beta$-lactamase. As will be discussed further below, it is important to note that while the final step is enzyme independent, the overall rate of product formation of course is not. The enzyme underwent rapid turnover with Dex-Cephem-Mtx, as evidenced by a $k_{cat}$ value of 190 ($\pm$10) $s^{-1}$ and a calculated $k_{cat}/K_m$ value of $3.5 \times 10^3$ $mM^{-1}$ $s^{-1}$. These values are similar to those seen with cephalothin and other cephalosporin antibiotics with cephem cores similar to that of Dex-Cephem-Mtx for the P99 $\beta$-lactamase. Somewhat surprisingly, expulsion of the Mtx-thiol was extremely slow, with $k_4$ on the order of $10^{-5}$ $s^{-1}$. To our knowledge, this rate constant is the slowest reported for expulsion of the C3′ leaving group from a cephalosporin.

The rate constant for the expulsion of the C3′ substituent has been determined for a number of cephalosporins. The rate of expulsion of the leaving group at the C3′ position, of course, depends both on the intrinsic reactivity of the C3′ substituent and the interaction of the molecule with the enzyme. Pratt has shown that for the thiophenol, pyridinium, and *N*,*N*-dimethylaniline-4-azo-2′-pyridinium leaving groups, expulsion of the C3′ leaving group is rate-determining and enzyme independent. Thus, these data can be compared to those reported here for Dex-Cephem-Mtx since all four reactions are enzyme independent. The $k_4$ values for these groups were reported to be 0.21, 0.44, and 11 $s^{-1}$, respectively (*31*, *35*). These rate constants are 4−6 orders higher in magnitude than that for Mtx-thiol. These rate constants are consistent with the relative p$K_a$'s of the leaving groups, although it has been shown that nucleofugality does not necessarily correlate with p$K_a$ (*37*). The p$K_a$ values for the thiophenol, pyridine, and *N*,*N*-dimethylaniline-4-azo-2′-pyridine were reported to be 6.43, 5.14, and 4.3, respectively (*31*, *38*). The p$K_a$ for the thiol group in Mtx-thiol would be expected to be that of a typical alkanethiol, ca. 11 (*39*). This comparison of the rate of expulsion of different C3′ leaving groups contributes to the general understanding of the influence of cephem structure on antibiotic resistance. These results not only have bearing on the future design of substrates for the chemical complementation assay, but also on molecules such as prodrugs and reporter substrates, which also depend on the rate of expulsion of the leaving group at the C3′ position (*40*, *41*).

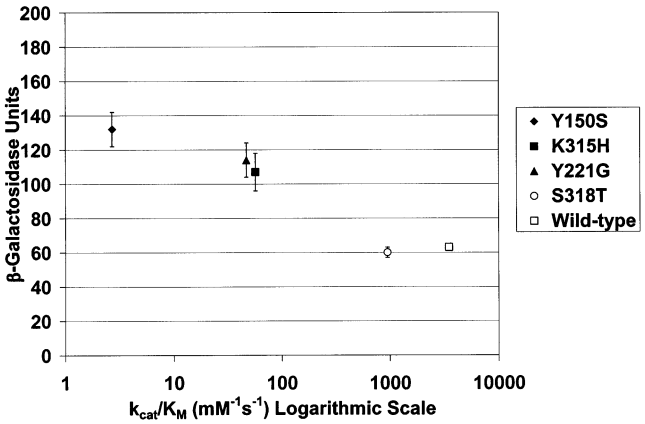



FIGURE 5: Correlation between catalytic efficiency and in vivo transcription of the wild-type P99 $\beta$-lactamase and its variants. Errors in $k_{cat}/K_m$ were negligible and not plotted. Standard deviations for *lacZ* transcription activation are shown. The standard deviation for the wild-type P99 is 2 units, and is subsumed by the symbol on the graph.

Having determined the kinetic pathway through which Dex-Cephem-Mtx is cleaved by the P99 $\beta$-lactamase, we could then assess if there is in fact a correlation between the catalytic efficiency of enzyme variants and their levels of transcription activation in the chemical complementation assay. There is a strong correlation between the catalytic efficiencies ($k_{cat}/K_m$) of the P99 $\beta$-lactamase variants and the levels of transcription activation in the chemical complementation assay (Figures 3 and 5, Table 3). As the activities of the P99 variants increase, the levels of transcription activation decrease, presumably because more of the Dex-Cephem-Mtx is cleaved. All of the variants examined here follow this trend, and the in vivo results agree with the kinetic scheme. Under the conditions used here, the dynamic range of the assay spans at least 3 orders of magnitude. The P99:S318T variant, which was down 1 order of magnitude in $k_{cat}/K_m$ from the wild-type enzyme, had a similar transcription read-out to that of the wild-type P99 $\beta$-lactamase. The two variants P99:K315H and P99:Y221G, which are both down 2 orders of magnitude, showed similar, intermediate levels of transcription activation. The P99:Y150S variant, which is down 3 orders of magnitude, had a similar transcription read-out to that of the inactive P99:S64A variant. Since the $K_m$s for all of the P99 variants examined here are very similar, it is not clear whether there is a better correlation between the $k_{cat}$ or the $k_{cat}/K_m$ and the transcription readout. This correlation supports the assumption that the P99 $\beta$-lactamase is acting on free Dex-Cephem-Mtx rather than the ternary complex in vivo.

As presently realized, the use of a *lacZ* reporter gene inherently limits the dynamic range. While the six mutants reported here span the full range of the *lacZ* assay, that range is only about 4-fold. This limitation to the *lacZ* dynamic range is intrinsic to *lacZ* activation in the yeast two-hybrid assay. As reported initially, the maximal levels of transcription activation with Dex-Cephem-Mtx in the yeast three-hybrid assay are only down 2-fold from that of a robust protein−protein interaction in the yeast two-hybrid assay (*14*). This activation is with a *lacZ* reporter gene with eight tandem LexA operators, the most sensitive *lacZ* reporter commercially available for the Brent two-hybrid system. To optimize the dynamic range, currently we are constructing

a yeast selection strain that will link bond cleavage to transcription of a *URA3* reporter gene (*42*). Such a reporter will allow a negative growth selection based on conversion of 5-fluoroorotic acid to 5-fluorouracil, which is toxic. Growth selections both allow small differences in activity to be distinguished based on exponential amplification (*43*) and sampling of a large dynamic range, by varying selection conditions—here, for example, by varying the concentration of 5-fluoroorotic acid in the growth reduction. For directed evolution applications ideally an assay would both be able to detect individual mutations that increase enzyme activity only a few-fold and be able to span several orders of magnitude in enzyme activity. The next step is to use a growth selection and a nonmodel system to optimize chemical complementation in both directions. The dynamic range of the assay can be further modulated by varying the enzyme concentration, the concentration of the transcriptional activator fusion proteins, the reporter gene, or other assay conditions.

It is interesting to speculate as to what determines the upper and lower limits of the sensitivity of the assay. Given that $k_4$ is the rate-determining step and is enzyme independent, the maximal activity that can be detected by the assay should be bound by $k_4$. To simplify, the reaction can be described as consisting of a sequence of two first-order reactions, the first with the rate constant $(k_{cat}/K_m)[E]$ for conversion of substrate to product 1 (III), the second with the rate constant $k_4$ for conversion of III to product 2 (V), in which the "intermediate" III builds up and later falls (*44*). As long as $(k_{cat}/K_m)[E] \gg k_4$, there should be a rapid depletion of I and build-up of III, and mutants with different $k_{cat}/K_m$'s should not affect the rate of V formation. It is only when $k_{cat}/K_m$ is reduced enough where $(k_{cat}/K_m)[E]$ approaches $k_4$ that mutants with different $k_{cat}/K_m$'s should be able to be distinguished. This analysis is consistent with the experimental data, within the uncertainty of assumptions about the activity and concentration of the enzyme variants inside the cell. For the wild-type enzyme, $k_{cat}/K_m$ is ca. $10^3$ $mM^{-1}$ $s^{-1}$, and the [E] in the cell is ca. $10^{-5}$ mM. Thus, $(k_{cat}/K_m)[E]$ is ca. $10^{-2}$ $s^{-1}$ and greater than $k_4$, which is ca. $10^{-5}$ $s^{-1}$. Thus, mutants with activity close to that of the wild-type enzyme, such as P99:S318T, have a similar read-out in the chemical complementation transcription assay because $(k_{cat}/K_m)[E] \gg k_4$ and there is still a rapid build-up of III from I. It is only mutants such as P99:K315H and P99:Y221G, where $k_{cat}/K_m$ is ca. $10^1$ $mM^{-1}$ $s^{-1}$ such that $(k_{cat}/K_m)[E]$ is ca. $10^{-4}$ $s^{-1}$ and approaching $k_4$, where there is no longer a rapid build-up of III that can be distinguished from the wild-type enzyme in this assay. Significantly, this upper limit is unique to the $\beta$-lactamase model system and should not limit future applications.

The minimal enzymatic activity that can be detected is determined by the amount of Dex-Cephem-Mtx that must be converted to Dex and Mtx product to affect the concentration of reconstituted transcriptional activator sufficiently to decrease the levels of transcription activation. The actual value is difficult to quantify because we do not know the true concentration of small molecule in the cell. This value is also more difficult to quantify because of the complexity of eukaryotic transcription activation. For example, the lifetime of the complex between the transcriptional activator and the Mediator promoter complex is not known (*45*). The interpretation is further complicated because not only does Dex-Cephem-Mtx dimerize the DBD−DHFR and AD−GR fusion proteins, but the hydrolyzed Dex and Mtx compete for binding to these fusion proteins. We favor a model in which the cell maintains a given concentration gradient of both substrate and product. To be detected in this assay, then, an enzyme must be able to convert substrate to product at a rate competitive with substrate influx and product efflux. While highly speculative, such a model is consistent with the observation that a single *lacZ* reporter is able to distinguish 3 orders of magnitude in enzyme activity. The most active variants tested here have a half-life on the order of seconds for conversion of substrate (I) to product (V), the variants with intermediate activity have a half-life on the order of hours, while the least active variant has a half-life on the order of a day.

Chemical complementation provides a general assay for enzyme catalysis that can be readily extended to new chemical reactions. In this approach, enzyme catalysis is linked to reporter gene transcription in vivo based on covalent coupling of two small molecule ligands. In the initial proof-of-principle paper, it was shown that the wild-type P99 $\beta$-lactamase could be isolated from a pool of inactive S64A variants using a *lacZ* screen. The strength of this approach, however, should be that the transcription read-out can distinguish enzymes based on their catalytic activity. Here we have shown that the catalytic efficiencies $(k_{cat}/K_m)$ of a series of P99 $\beta$-lactamase variants do in fact correlate with their overall levels of transcription in vivo in the chemical complementation assay. Although other general assays for enzyme catalysis have been reported recently, this work represents the first demonstration to our knowledge of an assay other than a microtiter plate assay where the read-out has been shown to correlate with enzyme activity. Using a *lacZ* screen, chemical complementation is able to distinguish $\beta$-lactamase variants that span 3 orders of magnitude in catalytic efficiency and that differ from one another by an order of magnitude. The dynamic range presumably can be further modulated by varying the reporter gene, the growth conditions, or the enzyme concentration. Having established the feasibility of chemical complementation with the $\beta$-lactamase model chemistry, this system can now be applied to new chemical reactions for which there is no natural screen or selection for function.

## ACKNOWLEDGMENT

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## SUPPORTING INFORMATION AVAILABLE

Michaelis-Menten plots of the wild-type P99 $\beta$-lactamase and its variants. $\beta$-Lactamase activity with the small molecule dimerizer, Dex-Cephem-Mtx, was measured for the wild-type $\beta$-lactamase and its variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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