NSC-16 cantilevers (resonance frequency ~180 kHz). Scan rates used were 1.5–2.5 Hz with a cantilever oscillation amplitude of the order of 20–40 nm.

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A Bacterial Small-Molecule Three-Hybrid System**

Eric A. Althoff and Virginia W. Cornish*

Affinity chromatography has long been used to identify the protein targets of small-molecule drugs and other biomolecules. Although an essential tool for biochemical research, affinity chromatography can often be labor-intensive and time-consuming. Recently, the yeast three-hybrid assay, a derivative of the two-hybrid assay, was introduced as a straightforward, in vivo alternative to affinity chromatography.[1, 2] In the three-hybrid assay, protein–small molecule interactions are detected by the dimerization of the two halves of a transcriptional activator (TA) through the receptors of the small molecule and subsequent transcription of a reporter gene.[3–6] For affinity chromatography applications, one ligand–receptor pair is used as an anchor and the other is the small molecule–protein interaction being investigated. Although the yeast three-hybrid assay is quite powerful, a bacterial equivalent would increase the number of proteins that could be tested by several orders of magnitude because the transformation efficiency of *E. coli* is significantly greater than that of *S. cerevisiae*. Furthermore, there may be applications where it is advantageous to test a eukaryotic protein in a prokaryotic environment in which many pathways are not conserved. However, the yeast three-hybrid assay cannot be transferred directly to bacteria. The components of the transcription machinery and the mechanism of transcriptional activation differ significantly between bacteria and yeast. Ligand–receptor pairs often are organism-specific because of cell permeability, toxicity, or other interactions with the cellular milieu. Bacterial two-hybrid assays have only begun to be developed in the past few years[7] and to date only initial efforts toward the design of a bacterial three-hybrid system have been reported.[8, 9] Herein we report the first robust small-molecule bacterial three-hybrid system—a heterodimer of methotrexate and a synthetic analogue of FK506 that activates transcription in the *E. coli* RNA polymerase two-hybrid system (Figure 1).

We chose to construct our bacterial three-hybrid system from the RNA polymerase two-hybrid system reported by Dove et al. in 1997.[10] A variety of methods for detecting protein–protein interactions in bacteria are now availa-

[*] Prof. V. W. Cornish, E. A. Althoff
Department of Chemistry, Columbia University
New York, NY 10027 (USA)
Fax: (+1) 212-932-1289
E-mail: vc114@columbia.edu

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

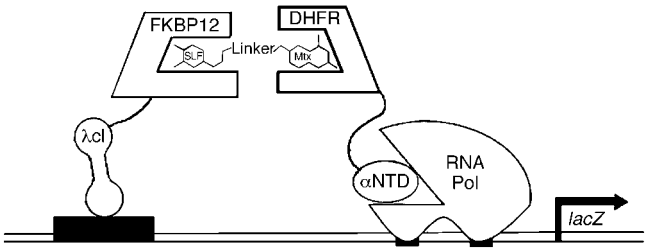
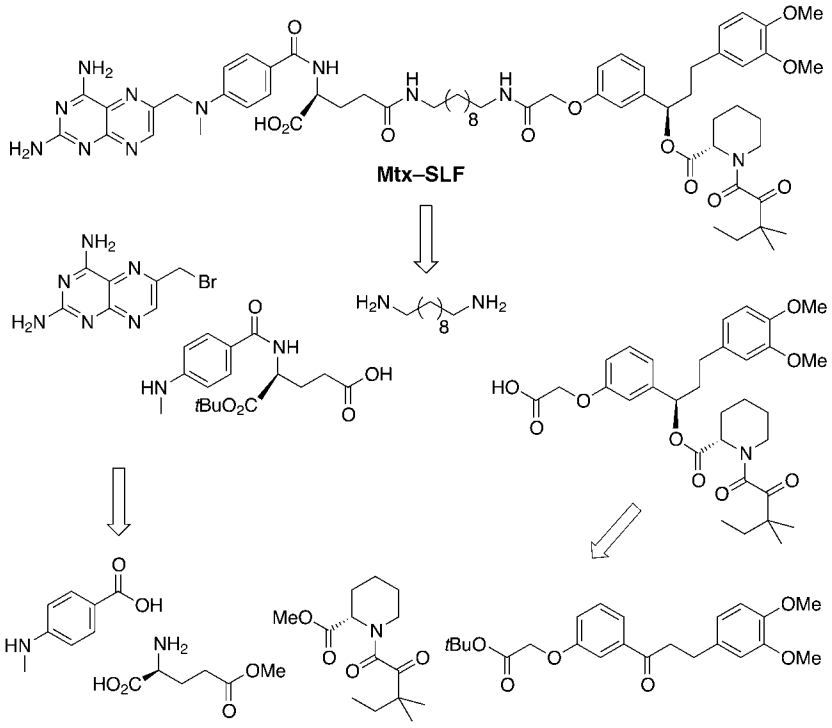


Figure 1. Bacterial RNA polymerase small-molecule three-hybrid system. A bacterial two-hybrid system was developed by Dove and Hochschild based on their observation that λcI-mediated recruitment of RNA polymerase (RNA Pol) is sufficient to activate gene transcription. Herein we report a three-hybrid variant of this system built around the interaction between the small molecules methotrexate (Mtx) and a synthetic analogue of FK506 (SLF) and their protein receptors, dihydrofolate reductase (DHFR) and FK506-binding protein 12 (FKBP12), respectively. In this assay, the binding site for the DNA-binding protein λcI is placed upstream of the promoter for a *lacZ* reporter gene. λcI is fused to FKBP12 and the N-terminal domain of the α-subunit of RNA Pol (αNTD) is fused to DHFR. Thus, upon addition of the small molecule heterodimer Mtx – SLF, the λcI – FKBP12 and αNTD – DHFR fusion proteins are dimerized, thus activating transcription of the *lacZ* reporter gene.

ble.[7–14] Generally, these methods are based either on enzyme complementation or transcriptional activation or repression assays. Whereas the enzyme complementation assays are essentially the same as those used in eukaryotes, entirely new transcription-based assays had to be developed for bacteria because the components of the transcription machinery are poorly conserved between eukaryotes and prokaryotes. We chose to adapt the RNA polymerase assay developed by Dove et al. because transcriptional activation in this assay results in a large increase in reporter gene transcription and because reconstitution of transcriptional activation should be largely conformation-independent. Based on their studies of the mechanism of transcriptional activation by λ-repressor (λcI),[15] Dove et al. developed an in vivo assay for protein – protein interactions based on dimerization of λcI and the N-terminal domain of the α-subunit of RNA polymerase (αNTD). They showed that this assay could detect the interaction between the proteins Gal4 and Gal11^P as an increase in transcription of a *lacZ* reporter gene.[16] We used this direct protein – protein interaction as a small-molecule independent positive control in work reported herein. Dove and Hochschild optimized the *lacZ* reporter gene such that dimerization of λcI and αNTD activates transcription 10 – 100-fold over basal levels. A variant of this assay that can be run as a *his3* growth selection was recently reported.[17]

The key to converting this two-hybrid assay into a three-hybrid system was the design of a dimeric ligand that could bridge λcI and αNTD through the receptors of the ligand. For the bridging small molecule, we chose to prepare a heterodimer of methotrexate (Mtx) and a synthetic analogue of FK506 (SLF). We call this heterodimer Mtx – SLF. We planned to use Mtx – SLF to dimerize a λcI – FKBP12 (λcI – FK506-binding protein 12) protein chimera and an αNTD – DHFR (αNTD – dihydrofolate reductase) protein chimera as shown in Figure 1. Mtx inhibits DHFR with a low picomolar K_I, and the interaction between the two has been extensively studied.[18, 19] Furthermore, our laboratory recently showed that Mtx could be used successfully in a yeast three-hybrid system.[6] For the other half of the bridging small molecule, we used SLF. FK506 functions as a natural small-molecule dimerizer, and dimers of FK506 have been developed as artificial chemical inducers of dimerization.[3, 20] SLF was developed by Ariad Pharmaceuticals as an FK506 analogue; it has a nanomolar affinity for FKBP12, and the interaction between the two has been fully characterized.[21, 22] Furthermore, SLF homodimers have been used previously in several mammalian three-hybrid systems.[23]

The retrosynthetic analysis of Mtx – SLF is shown in Scheme 1. The synthesis is based on previous syntheses of Mtx and SLF derivatives and was designed to allow Mtx, SLF, or the linker between them to be varied readily. The Mtx portion of the molecule begins as the γ-methyl ester of L-glutamic acid and is based on previous syntheses of Mtx.[6, 24] γ-Methyl L-glutamic acid is inexpensive, and the α-carboxylate can be selectively protected as the *tert*-butyl ester by transiently protonating the α-amino group.[25] The diprotected amino acid is then coupled to 4-(methylamino)benzoic acid by using standard peptide coupling reagents. Finally, the γ-methyl ester is saponified to yield the free acid for further reactions. SLF acid was synthesized as described previously from L-pipecolinic acid in 59% yield over six steps.[5, 21] The Mtx and SLF portions were then coupled to 1,10-diaminodecane in a three-component peptide coupling reaction. 2,4-



Scheme 1. Retrosynthetic analysis of Mtx – SLF.

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Diamino-6-bromomethyl-pteridine is added after this coupling reaction to simplify the purification of the synthetic intermediates. Finally, acid cleavage of the *tert*-butyl ester yielded Mtx–SLF. Thus, the Mtx–SLF heterodimer was prepared from two components in 5% overall yield over the six steps from the γ-methyl ester of L-glutamic acid or 6% overall yield in nine steps from the L-pipecolinic acid precursor of SLF.

The next step was the construction of the *E. coli* strain that expresses the λcI–FKBP12 and αNTD–DHFR fusion proteins and contains the *lacZ* reporter construct. Plasmids that encode the λcI–FKBP12 and αNTD–DHFR chimeras were prepared from vectors pACλcI32 and pBRαLN by using standard molecular biology techniques.[7] We used the same synthetic *lacZ* reporter, p*lac*OR2-62, initially reported by the Hochschild group. The reporter p*lac*OR2-62 is maintained in one copy in the chromosome as a prophage and encodes the *lac*Z gene 62 base pairs downstream from the λcI binding site (Figure 1).[10] Based on previous results from Kopytek and Hu showing that *tolC*$^-$, which encodes a portion of a multidrug-resistance efflux pump (MDR), and *thyA*$^-$, which encodes an enzyme upstream of DHFR in the thymidine biosynthesis pathway, mutations improved the viability and tolerance of *E. coli* to Mtx-based molecules, we expected export as well as toxicity of Mtx–SLF to be problematic in *E. coli*.[26] Thus, we modified the original Hochschild strain KS1 to be *tolC*$^-$ to decrease active export of our small molecule by the TolC-dependent MDR efflux pump. At the low concentrations of Mtx–SLF required for the three-hybrid experiments, however, Mtx was not sufficiently toxic to warrant the *thyA*$^-$ mutation (data not shown). We introduced the *tolC*$^-$ mutation into KS1 by using a standard molecular biology technique for gene transfer, P1*vir* transduction, from strain SK037.[26] We call this test strain V674E. Transformation of the plasmids bearing the various λcI and αNTD fusion proteins into V674E yielded the final experimental strains.

We used standard β-galactosidase assays in liquid culture and on plates[27] to establish that Mtx–SLF activates transcription of the *lacZ* reporter gene in *E. coli* strain V674E (Figure 2 and Supporting Information). Overnight cultures were used to innoculate fresh LB media that contained Mtx–SLF, IPTG, and the appropriate antibiotics. These cultures were grown, lysed, and assayed for β-galactosidase activity by using *o*-nitrophenyl-β-D-galactopyranoside (ONPG), a chromogenic substrate for β-galactosidase. Cells that express λcI–FKBP12 and αNTD–DHFR showed sixfold greater activation of *lacZ* transcription at 1 μM Mtx–SLF and tenfold greater activation at 10 μM Mtx–SLF than cells that express only λcI and αNTD. For comparison, the levels of transcription activation for the direct protein–protein interaction are 13-fold higher and are unaffected by the concentration of Mtx–SLF in the media. As seen in Figure 3, the levels of transcriptional activation in the three-hybrid system correlate with the concentration of Mtx–SLF in the media. We begin to see transcriptional activation at 0.3 μM Mtx–SLF, and the levels of activation are still increasing at 10 μM Mtx–SLF. Concentrations higher than 10 μM Mtx–SLF cannot be used as the small molecule begins to become toxic to the *E. coli*. Several independent controls establish that transcriptional activation indeed requires both halves of Mtx–SLF (Figure 2). Neither Mtx, SLF, nor a combination of the two increases the levels of transcription in the three-hybrid system (data not shown). At 1 μM Mtx–SLF, a tenfold excess of either Mtx or the *tert*-butyl ester of SLF decreased the levels of transcription to about half that with 1 μM Mtx–SLF alone. Deletion of either DHFR or FKBP12 from the λcI–FKBP12 and αNTD–DHFR fusion proteins drops the levels of small-molecule-induced transcriptional activation to the background levels observed in cells that express only λcI and αNTD.

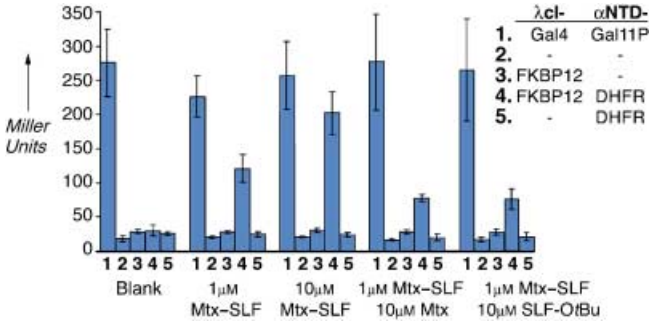

Figure 2. The levels of small-molecule-induced transcriptional activation were quantified by using liquid *lacZ* assays. The strains are assayed in liquid ONPG assays in which the levels of transcriptional activation can be quantified based on the amount of reporter protein β-galactosidase that is produced. Each column corresponds to strain V674E bearing plasmids expressing various λcI and αNTD fusion proteins: **1**, λcI–Gal4, αNTD–Gal11^P; **2**, λcI, αNTD; **3**, λcI–FKBP12, αNTD; **4**, λcI–FKBP12, αNTD–DHFR; **5**, λcI, αNTD–DHFR. Strain **1** is the Gal4–Gal11^P direct protein–protein interaction used as a positive control. Strains **2**, **3**, and **5** lack either DHFR or FKBP12 or both and are used as negative controls to test the assumption that transcriptional activation is dependent on both halves of the Mtx–SLF small molecule. The last two small-molecule concentrations are competition assays in which an excess of one of the ligands for the receptor proteins was used to compete out the positive signal by effectively decreasing the number of successful three-hybrid interactions. The strains were assayed in triplicate from three transformants and standard deviations are shown. The strains are grown in LB medium with IPTG (0.5 mM), ampicillin (100 μg mL^{-1}), chloramphenicol (6 μg mL^{-1}), and small molecules at the indicated concentration.

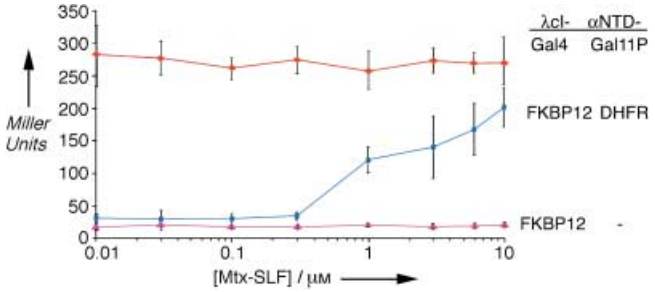

Figure 3. The levels of transcriptional activation depend on the concentration of Mtx–SLF in the bacterial three-hybrid system. The concentrations of Mtx–SLF in the media were varied, and the levels of *lacZ* transcription were quantified in liquid culture by using ONPG. The strains are V674E expressing the following λcI and αNTD fusion proteins: (◆), λcI–Gal4, αNTD–Gal11^P (a direct protein–protein interaction); (▲), λcI–FKBP12, αNTD (a negative control); and (■), λcI–FKBP12, αNTD–DHFR (the three-hybrid system). The rate of ONPG hydrolysis was measured in triplicate from three different transformants after growth in LB media that contained IPTG (0.5 mM), ampicillin (100 μg mL^{-1}), chloramphenicol (6 μg mL^{-1}), and Mtx–SLF at the indicated concentrations. The standard deviation for each data point is also shown.

The bacterial small-molecule three-hybrid system described herein should provide a robust platform for high-throughput assays based on protein–small molecule interactions. The Mtx–SLF heterodimeric ligand can be prepared readily and gives a strong transcription readout in the *E. coli* RNA polymerase three-hybrid system. Notably, the levels of transcriptional activation with the Mtx–SLF three-hybrid system are comparable to those with the direct protein–protein interaction, despite the fact that one noncovalent interaction has been replaced with two. The EC_{50} for *lacZ* transcription is greater than the K_D of either Mtx or SLF for FKBP12.[5] Currently we are carrying out in vitro experiments to examine the relationship between *lac*Z transcription and the K_D of the ligand–receptor interaction. Three-hybrid systems provide an in vivo alternative to affinity chromatography that can be used to evolve proteins that recognize a particular small molecule, to screen a library of small molecules based on binding to a particular protein, or to screen cDNA libraries to find the protein targets of drugs or to classify proteins based on their small-molecule interactions. Because of the high transformation efficiency and rapid doubling time of *E. coli*, this system should increase the number of proteins that can be tested in three-hybrid assays by several orders of magnitude compared with yeast systems. A bacterial assay should be particularly advantageous in molecular evolution experiments in which in the order of 10^8 variants may be necessary to alter protein function. Based on our results, we believe that Mtx will provide a versatile anchor for presenting a variety of different small molecules.

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Proton-Induced, Reversible Evolution of O_2 from the Os^{IV}–Sulfoximido Complex $[Os^{IV}(tpy)(Cl)_2\{NS(O)\text{-}3,5\text{-}Me_2C_6H_3\}]$**

My Hang V. Huynh,* David E. Morris, Peter S. White, and Thomas J. Meyer*

O_2 activation in biological systems is a key step in respiration with O_2 activation achieved by a complex series of steps involving binding to an Fe–heme, electron transfer, and, ultimately, atom transfer to a reducing substrate.[1] Kinetic difficulties in the electroreduction of O_2 to H_2O in fuel cells create a significant over-voltage which limits

[*] Dr. T. J. Meyer
Associate Director for Strategic Research
Los Alamos National Laboratory, MS A127
Los Alamos, NM 87545 (USA)
Fax: (+1) 505-667-5450
E-mail: tjmeyer@lanl.gov

Dr. M. H. V. Huynh
Director-Funded Postdoctoral Fellow
Los Alamos National Laboratory
Chemistry Division MS J514
Los Alamos, NM 87545 (USA)
Fax: (+1) 505-667-3314
E-mail: huynh@lanl.gov

Dr. D. E. Morris
Actinide, Catalysis, and Separations Group
Chemistry Division MS J514
and the Glenn T. Seaborg Institute for Transactinium Science
Los Alamos National Laboratory
Los Alamos, NM 87545 (USA)

Dr. P. S. White
Department of Chemistry
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-3290 (USA)

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