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A Yeast Three Hybrid Assay for Metabolic Engineering of Tetracycline Derivatives

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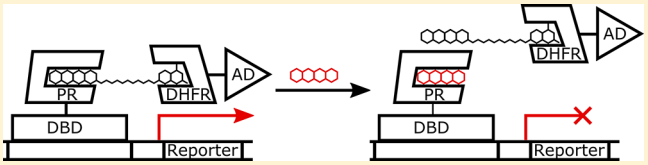
Supporting Information

ABSTRACT: Metabolic engineering stands to transform the discovery and production of a wide range of chemicals, but metabolic engineering currently demands considerable resource investments that restrict commercial application. To facilitate the applicability of metabolic engineering, general high-throughput and readily implemented technologies are needed to assay vast libraries of strains producing desirable chemicals. Toward this end, we describe here the development of a yeast three hybrid (Y3H) assay as a general, high-throughput, versatile and readily implemented approach for the detection of target molecule biosynthesis. Our system detects target molecule biosynthesis through a change in reporter gene transcription that results from the binding of the target molecule to a modular protein receptor. We demonstrate the use of the Y3H assay for detecting the biosynthesis of tetracyclines, a major class of antibiotics, based on the interaction between tetracyclines and the tetracycline repressor protein (TetR). Various tetracycline derivatives can be detected using our assay, whose versatility enables its use both as a screen and a selection to match the needs and instrumentation of a wide range of end users. We demonstrate the applicability of the Y3H assay to metabolic engineering by differentiating between producer and nonproducer strains of the natural product tetracycline TAN-1612. The Y3H assay is superior to state-of-the-art HPLC-MS methods in throughput and limit of detection of tetracycline derivatives. Finally, our establishment of the Y3H assay for detecting the biosynthesis of a tetracycline supports the generality of the Y3H assay for detecting the biosynthesis of many other target molecules.



Metabolic engineering is a potentially greener and more economical approach than traditional organic synthesis for the production of a wide range of organic chemicals. As opposed to organic synthesis, metabolic engineering is capable of producing valuable chemicals from readily available and renewable carbon sources and significantly reducing the use of organic solvents and reagents.[1,2] Traditional methods in DNA mutagenesis, advances in DNA synthesis, DNA assembly, and genome engineering are enabling high-throughput strain construction in metabolic engineering.[3−8] High-throughput strain construction is necessary because not only the multigene biosynthetic pathway for the natural product but also the underlying metabolism of the host must be optimized.[9] Assuming a biosynthetic pathway of 5 genes and 10 interacting host genes to test just 5 variants for each of these genes results already in a library size exceeding 10^{10}. However, the throughput of the most highly employed assaying methods such as LC-MS is only ~10^2 to 10^3 samples per day and is thus heavily limiting in assaying strains for successful metabolic engineering.[5,9]

The currently employed methods for assaying metabolite production in metabolic engineering are either low-throughput and general or high-throughput but not general. LC-MS methods are general but low-throughput, enabling the screening of only about 10^2 to 10^3 samples per day.[9] Colorimetric, fluorometric, and growth assays for molecules that are colored, fluorescent, essential for producer strain growth or could be easily converted to one of these, are high-throughput but not general.[10] Attempts have been made to develop assays that are both high-throughput and general, but it is yet to be determined whether or not these assays could be widely implemented.[10,11] Versatile, general, readily implemented high-throughput assays are required to transform metabolic engineering from a field with a high potential to a field with an extensive real-world impact on the way chemicals are made.

We set out to adapt the Y3H assay, previously employed for various basic science applications by our laboratory and others,[12−18] to a general, readily implemented, high-throughput versatile assay for metabolic engineering. We wished to develop an assay that can be readily adapted to new target molecules, used as either a screen or a selection, and minimizes

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handling, to match varying needs and instrumentation availabilities of end users.

Here, we demonstrate the application of the Y3H assay to detect the biosynthesis of tetracyclines, one of the major classes of antibiotics. We describe the engineering of the Y3H system to detect tetracyclines by synthesizing a minocycline-methotrexate chemical inducer of dimerization (CID) and cloning a LexA-TetR (tetracycline repressor) fusion protein.[14] Reporter gene expression responds to tetracycline in our system by competitive binding of tetracycline and the CID to the LexA-TetR protein fusion (Figure 1). Our assay can report

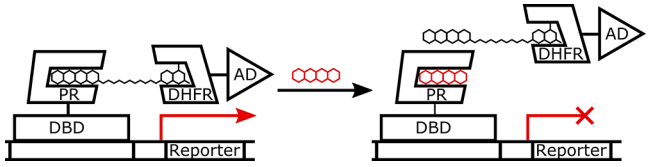

Figure 1. Detection of a target molecule biosynthesis by the yeast three hybrid system. Low/no production of a target molecule is detected by high reporter gene expression (left). High production titers of the target molecule outcompete the chemical inducer of dimerization (CID) from the fusion protein receptor and are detected by lower gene expression (right). DBD = DNA binding domain (e.g., LexA); AD = activation domain (e.g., B42); PR = protein receptor (e.g., TetR); DHFR = dihydrofolate reductase.

the presence of tetracyclines using various reporter gene outputs, enabling the use of the assay both as a screen and a selection to match the needs and instrumentation of a variety of end users. We show the applicability of our assay to metabolic engineering through the differentiation between producer and nonproducer strains of the natural product tetracycline, TAN-1612.

MATERIALS AND METHODS

General methods. Absorption and fluorescence spectra were recorded on an Infinite-M200 fluorescent spectrometer. DNA sequences were purchased from IDT. Polymerases, restriction enzymes, and Gibson Assembly mix were purchased from New England Biolabs. Sanger sequencing was performed by Genewiz. Yeast strains were grown at 30 °C, and the shaker setting was 200 rpm, unless otherwise indicated. Yeast transformations were done using the lithium acetate method.[19] Plasmids were cloned and amplified using Gibson Assembly and cloning strain C3040 (New England Biolabs).[20] Unless otherwise indicated, yeast strains were grown on synthetic minimal media lacking histidine and/or uracil and/or tryptophan and/or leucine, as indicated by the abbreviation HUTL$^-$.[21] Tables S1 and S2 list the strains and plasmids used in this study. Yeast strain patches were obtained from glycerol stocks by streaking on an agar plate of synthetic medium lacking the appropriate amino acid markers, incubating at 30 °C for 3 days, patching single colonies onto a fresh agar plate, and incubating at 30 °C overnight. Unless otherwise indicated, when 96-well plates were placed in the yeast shaker, 95% of the interface between the plate and its cover was wrapped with laboratory film (Parafilm, Bemis PM999). All Y3H assays were performed with sterile components and three biological replicates.

All chemical reactions were carried out under an argon atmosphere with anhydrous solvents unless otherwise indicated. Analytical high-pressure liquid chromatography (HPLC) was performed with a C-18 column, 2.6 μ, 250 mm × 4.6 mm, with the eluent given in parentheses. Unless stated otherwise, reactions were monitored using analytical HPLC with a separation gradient of 10−90% MeCN in 0.1% (v/v) TFA aqueous solution over 20 min. Preparative HPLC was carried out with a C-18 5 μ column, 250 mm × 10 mm, with the eluent given in parentheses. NMR spectra were obtained using Bruker 400 or 500 MHz instruments, as indicated. Unless stated otherwise, mass spectroscopy measurements were performed on an Advion CMS mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources. TAN-1612 quantification was performed using a phenyl-hexyl 1.7 μ 100 mm × 3 mm column, on a Waters SQD2 quadrupole mass spectrometer equipped with a UPC2 SFC inlet, a photodiode array (PDA) UV−vis detector, and a dual ESI/APCI probe. Unless stated otherwise, all reagents, salts, and solvents were purchased from commercial sources and used without further purification.

*Synthesis of the Min-Mtx CID (**1**, Scheme 1). Step A.* To 9-NH_2-minocycline (100 mg, 0.2 mmol), Boc-11-aminoundecanoic acid (89 mg, 0.3 mmol), EDC (75 mg, 0.4 mmol), and HOBt (60 mg, 0.4) were added DMF (1 mL) and Et_3N (139 μL, 1.0 mmol), and the reaction was mixed at rt for 6 h. The reaction progress was monitored by RP-HPLC. Aqueous $NaHCO_3$ (0.05 M)[22] was then added, and the aqueous phase was extracted three times with $CHCl_3$. The combined organic fraction was extracted with brine, and the combined brine extracts were extracted twice with $CHCl_3$. The combined organic fraction was dried with Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified in two batches by preparative RP-HPLC (30−35% MeCN in 99.9%:0.1% H_2O/TFA, 45 min) to afford compound **3** (53.4 mg, 36%) as a yellow solid. ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.60 (s, 1H), 4.11 (s, 1H), 3.24 (dd, J = 15.4, 4.2, 1H), 3.14 (s, 6H), 3.08 (m, 3H), 3.02 (s, 6H), 2.50 (t, J = 7.4, 2H), 2.44 (t, J = 14.3, 1H), 2.24 (m, 1H), 1.72 (m, 1H), 1.62 (m, 1H), 1.43 (s, 9H), 1.42 (m, 4H), 1.38 (m, 2H), 1.32 (m, 10H). MS (APCI+): m/z calcd for $C_{39}H_{58}N_5O_{10}^+$, 756.42; found, 756.4 $[M + H]^+$. MS (APCI−): m/z calcd for $C_{39}H_{56}N_5O_{10}^-$, 754.40; found, 754.3 $[M - H]^-$.

Steps B−D. To compound **3** (27.3 mg, 0.036 mmol) were added DCM (0.5 mL) and TFA (0.5 mL), and the reaction was mixed at rt for 12 min. The solvent was removed under reduced pressure. PhMe was added, and the solvent was again removed under reduced pressure. PhMe addition and solvent removal was repeated once more. Methotrexate-α-OtBu (27.5 mg, 0.054 mmol)[23,24] and PyBOP (37.5 mg, 0.072 mmol) were added, followed by DIPEA (11.5 mg, 0.09 mmol) in DMF (1 mL), and the reaction was mixed at rt. After 22 h, EtOAc and aqueous $NaHCO_3$ were added, and the aqueous phase was extracted twice with EtOAc. The combined organic fraction was extracted with brine, and the brine extract was filtered. The solid residue was redissolved in MeOH, and the solvent was removed under reduced pressure. The crude product was purified in two batches by preparative RP-HPLC (20−30% MeCN in 99.9%:0.1% H_2O/TFA, 50 min). Following solvent removal under reduced pressure, TFA (2 mL) was added and the reaction was stirred at rt for 90 min. The reaction progress was monitored by RP-HPLC. The solvent was removed under reduced pressure to afford compound **1** (4.1 mg, 10%) as a yellow solid. ^{1}H NMR (500 MHz, MeOD-d_4): δ 8.64 (s, 1H), 8.25 (s, 1H), 7.76 (d, J = 8.8,

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2H), 6.86 (d, J = 8.8, 2H), 4.92 (s, 2H), 4.54 (dd, J = 8.8, 4.5, 1H), 4.08 (s, 1H), 3.27 (s, 3H), 3.11 (m, 2H), 3.00 (s, 6H), 2.83 (s, 3H), 2.80, (s, 3H), 2.47 (m, 2H), 2.41−2.05 (m, 6H), 1.70−1.59 (m, 4H), 1.48−1.28 (m, 16H). MS (ESI+): m/z calcd for $C_{54}H_{71}N_{13}O_{12}^{2+}$, 1093.54; found, 546.9 $[M + 2H]^{2+}$.

Y3H Strain Construction. LexA-TetR Plasmids. Plasmids PBA-Gib5 and HL-249−1 through HL-249−6 encoding LexA-TetR protein fusions for the various TetR classes were cloned by Gibson Assembly of the appropriate TetR sequence with pMW103 digested with restriction enzymes *Bam*HI-HF and *Eco*RI-HF (Table S3). All TetR sequences with the exception of TetR(B) were obtained as IDT gBlocks. TetR(B) sequence was amplified from pET21d-TetR, courtesy of A. Davidson.[25,26]

LacZ Assay Strain Construction. V506 was grown overnight in UT$^-$ media.[27] The culture was then diluted 10^4−10^5× with UT$^-$ and plated on UT$^-$ plates. Single colonies were streaked on HTU$^-$ and UT$^-$ plates and a streak growing on UT$^-$ but not on HTU$^-$, indicating the loss of the pMW3-(GSG)2rGR2 plasmid, was glycerol stocked as PBA5. This strain was further used for the transformation of the LexA-TetR plasmids to generate LacZ assay strains PBA8 and HL-260−1 through HL-260−7.

Growth Assay Strain Construction. Yeast strain LW2635[28] was transformed with plasmid PBA-Gib5 to generate strain PBA6. PBA6 was transformed with pMW2eDHFR[27] to generate the Y3H growth assay strain PBA14.

mCherry Reporter Plasmid. Reporter plasmid HL-174−2 encoding mCherry under the transcriptional control of LexA operators was cloned by Gibson Assembly of an mCherry sequence amplified from yEpGAP-Cherry[29] and an 8LexA-min-pGal1 sequence amplified from pMW112[14] into pRS416 digested with SacI and XhoI.

Fluorescent Protein Assay Strain Construction. V506 was grown overnight in T$^-$ media. The culture was then diluted 10^4−10^5× with T$^-$ and plated on T$^-$ plates. Single colonies were streaked on TU$^-$, HT$^-$, and T$^-$ plates and a streak growing on T$^-$ but not on UT$^-$ or on HT$^-$, indicating the loss of the pMW106 and pMW3(GSG)2rGR2 plasmids, was glycerol stocked as PBA3. This strain was transformed with plasmid HL-174−2 to generate yeast strain HL-294−7. The latter was transformed with PBA-Gib5 to generate the Y3H fluorescent reporter assay strain HL-2−4−1.

TAN-1612 Producer/Nonproducer Strain Construction. TAN-1612 producer and nonproducer strains EH-3−54−4 and EH-3−54−2 were obtained by transforming plasmids pYR342 and pYR291 or pYR342 and pRS426, respectively, into yeast strain BJ5464-NpgA (Tables S1 and S2).[30]

Protocol for Fluorescent Protein Y3H Assay. Fresh patches of Y3H strain HL-2−4−1 were inoculated into HTU$^-$ media (1 mL) in 24-well plates (flat, clear bottom, Corning no. 3526) and placed in the shaker. After 24 h, the cells were pelleted by centrifugation at 3250 rpm and resuspended in H_2O (1 mL). Pelleting and resuspension were repeated once more. OD_{600} was measured by diluting 10× into H_2O, and the cells were diluted accordingly into H_2O for OD_{600} = 1 to be used as a 10× solution. A total of 20 μL of cells of OD_{600} = 1 were added to the assay plate as the last component in the assay plate (flat, clear bottom, black 96-well plate, Corning no. 3603). Each well of the assay plate contained 200 μL total liquid volume, 20 μL cells, 100 μL of 2× HTU$^-$ media of 4% raffinose and no glucose, 20 μL of 10× 20% galactose aqueous solution, 20 μL of 10× CID solution of 100 μM in 10% DMSO aqueous solution, 20 μL supernatant and 20 μL purified TAN-1612 in a 10% DMSO aqueous solution,[30] or a 10% DMSO aqueous solution. Starting conditions in the assay plate were OD_{600} = 0.1, 2% galactose, 2% raffinose, 0% glucose, 1× HTU$^-$, 10 μM CID, 2% DMSO. The assay plate was placed in the 30 °C shaker overnight before measuring OD_{600} and 620 nm fluorescence (λ_{ex} = 588 nm).[31]

TAN-1612 supernatants from flask cultures were obtained by inoculating TAN-1612 producing and nonproducing strains, EH-3−54−4 and EH-3−54−2, respectively, in UT$^-$ media (5 mL) in 15 mL culture tubes (Corning 352059) and placing in the shaker overnight. Overnight cultures were used to inoculate 100 mL of YPD in 500 mL conical flasks with a starting OD_{600} of 0.08. After 68 h, 1 mL of culture was pelleted in 1.5 mL Eppendorf tubes at 14 000 rpm, sterile filtered, and diluted 20× into H_2O to be used as a 10× stock solution.

TAN-1612 supernatants from 96-well plate cultures were obtained by inoculating 12 and 84 colonies of TAN-1612 producing and nonproducing strains, EH-3−54−4 and EH-3−54−2, respectively, in UT$^-$ media (0.5 mL) in a 96-well plate (Corning P-2 ML-SQ−C-S). The plate was covered with two layers of SealMate film (Excel Scientific, SM-KIT-BS) and placed in a shaker overnight. Overnight cultures were used to inoculate 300 μL YPD in an identical setup with a starting OD_{600} of 0.1 and placed in an 800 rpm shaker overnight. After 54 h, 150 μL of H_2O was added to the cultures to prevent drying of cultures due to evaporation and two new layers of Sealmate film were replaced. After 78 h, the cultures were pelleted at 3250 rpm, and the supernatant was diluted 20× into H_2O to be used as a 10× stock solution.

Protocol for Y3H Growth Assay. Fresh patches of Y3H strain PBA14 were inoculated in HT$^-$ media (1 mL) and placed in the shaker overnight. Cultures were then centrifuged at 3000 rpm for 3 min, and the pellets were resuspended with H_2O (1 mL). Pelleting and resuspension was repeated twice more. OD_{600} was measured, and the cells were diluted accordingly into H_2O to OD_{600} = 1 to be used as a 10× stock solution. A total of 20 μL of cells of OD_{600} = 1 were added as the last component to the assay plate (flat, clear bottom, black 96-well plate, Corning no. 3603). Each well of the assay plate contained 200 μL total liquid volume, 20 μL cells, 100 μL of 2× HT$^-$ media with 4% raffinose, 4% galactose, 0% glucose, 0.4% 5-FOA, 20 μL of 0/250 μM 10× CID in 10% DMSO aqueous solution, 20 μL of doxycycline (0/50 μM in 10% EtOH aqueous solution), and 40 μL of H_2O. Starting conditions in the assay plate were OD_{600} = 0.1, 2% galactose, 2% raffinose, 0% glucose, 0.2% 5-FOA, 1× HT$^-$, 25 μM CID, 1% DMSO, 1% EtOH. The assay plate was placed in the 30 °C shaker for 5 days with daily measurements of OD_{600}.

Protocol for Y3H LacZ Assay. Fresh patches of Y3H strains PBA-8 and HL-260−1 through HL-260−7 were inoculated into HTU$^-$ media (1 mL) in 24-well plates (flat, clear bottom, Corning no. 3526) and place at 30 °C shaker. After 24 h, the cells were pelleted by centrifugation at 3000 rpm for 5 min and resuspended in H_2O (1 mL). Pelleting and resuspension were repeated once more. OD_{600} was measured, and the cells were diluted accordingly into H_2O to OD_{600} = 1 to be used as a 10× solution. A total of 20 μL of cells of OD_{600} = 1 were added to the assay plate as the last component in the assay plate (clear v-bottom, clear 96-well plate, Corning no. 3894). Each well of the assay plate contained 200 μL total liquid volume, 20 μL of cells, 100 μL of 2× HTU$^-$ media of 4% raffinose and no glucose, 20 μL of 10× 20% galactose aqueous solution, 20 μL

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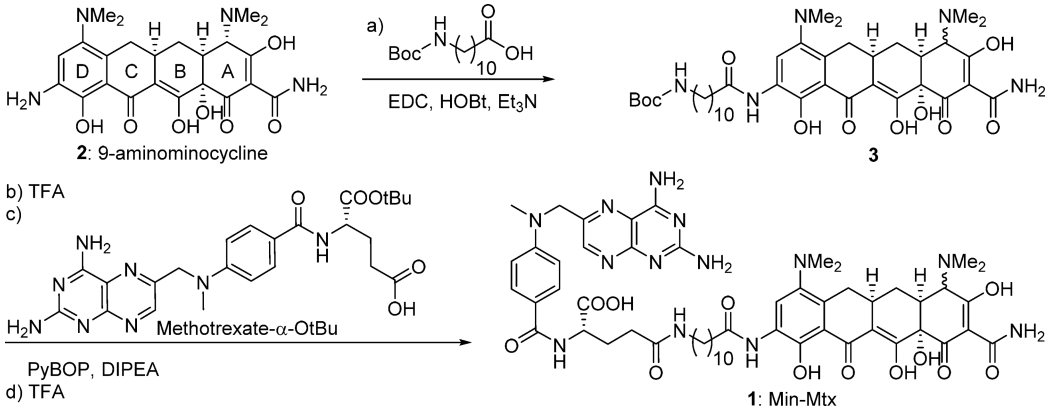
Scheme 1. Synthesis of the Chemical Inducer of Dimerization (CID) Min-Mtx[a]



[a]Reagents and conditions: (a) Boc-11-aminoundecanoic acid (1.5 equiv), EDC (2 equiv), HOBt (2 equiv), Et_3N (5 equiv), DMF, 22 °C, 6 h, 36%; (b) TFA/DCM (1:1), 22 °C, 12 min; (c) methotrexate-α-OtBu (1.5 equiv),[23,24] PYBOP (2 equiv), DIPEA (2.5 equiv), DMF, 22 °C, 22 h; (d) TFA, 22 °C, 1.5 h, 10% over three steps. EDC = *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole, TFA = trifluoroacetic acid, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, DIPEA = *N*-ethyldiisopropylamine.

of 10× CID solution in 10% DMSO aqueous solution or a 10% DMSO aqueous solution, 20 μL of the tetracycline ligand in 10% EtOH aqueous solution, and 20 μL of water. Starting conditions in the assay plate are OD_{600} = 0.1, 2% galactose, 2% raffinose, 0% glucose, 1× HTU^-, varying concentration CID, varying concentration of tetracycline ligand, 1% DMSO, and 1% EtOH. The assay plate was placed in the shaker overnight. OD measurements were then taken after 24 h, and the cells were pelleted and resuspended in 100 μL of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 2 mM $MgSO_4$, pH adjusted to 7, 2.7 mL/L of β-mercaptoethanol added fresh the day of the assay). The cells were pelleted again, resuspended in 100 μL of Y-PER buffer (Fisher Scientific, no. 78990), and lysed for 30 min at rt before the addition of *o*-nitrophenyl β-D-galactopyranoside (ONPG) in Z-buffer (8.5 μL, 10 mg/mL). After 90 min, a Na_2CO_3 solution (1 M, 110 μL) was added to quench the reaction. After pelleting at 3000 rpm, 175 μL of the supernatant was transferred into flat clear-bottom 96-well plates (Corning no. 353072), and OD_{420} was measured. The limit of detection was calculated as the concentration of molecule at which the signal-to-noise ratio of the assay was >3:1.[32,33]

RESULTS

For a Y3H system detecting tetracyclines, we cloned a Y3H strain encoding LexA-TetR and DHFR-B42 fusion proteins and synthesized a minocycline-methotrexate (Min-Mtx) CID (Figure 1). We chose to base our Y3H system on well-studied components, namely, the LexA DNA binding domain (DBD) and B42 activation domain (AD),[34] respectively, introduced by Brent and co-workers,[14,27,35,36] as well as the DHFR-methotrexate and TetR-tetracycline interactions. The latter two are highly characterized receptor−ligand interactions of picomolar affinity,[37] and the methotrexate-DFHR interaction has been used numerous times by our laboratory.[27,35] We used LexA-TetR fusion proteins of TetR variants that were highly studied, such as the variants from classes B and D,[37−39] as well TetR variants from the much less studied TetR classes A,[40,41] C,[42,43] E,[44,45] G,[46] and H.[47−50] TetR and LexA form stable homodimers, with each of the TetR monomers binding tetracyclines and LexA binding DNA as a dimer.[38,51] Thus, it is possible that the monomer ratio of TetR/LexA in the LexA-TetR fusion protein is unequal to 1, affecting accordingly the local concentrations of the Min-Mtx CID and DHFR-B42 as well. In any case, increased levels of tetracyclines are expected to outcompete the CID from the LexA-TetR protein fusion and lower reporter protein expression (Figure 1).

Our design and synthesis of the Min-Mtx CID is based on the wealth of the structure−activity relationship (SAR) data for the interaction of tetracyclines with TetR, as well as our previously reported CIDs.[27,35] It is known from inspection of the high-resolution structure of TetR bound to tetracycline derivatives as well as biochemical characterization that tetracyclines can be derivatized at the D ring without disrupting binding to TetR (Scheme 1).[37,38,52] Moreover, the FDA-approved analogue tigecycline and the clinical candidates eravacycline and TP-271 are all 9-amidotetracyclines;[53,54] thus, synthesis of a 9-amidotetracycline CID was logical. Specifically, 9-aminominocycline was chosen as the starting material because of its high acid stability relative to tetracycline as well as its commercial availability.[55] The design and synthesis of the Mtx component of the Min-Mtx CID was based on our previously reported methotrexate CIDs (Scheme 1).[27,35]

With the Y3H strains and Min-Mtx CID in hand, we verified the functionality of our Y3H system and determined the optimal CID concentration and TetR variant to further use in the assay. First, we confirmed that reporter gene output in our system is dependent upon the presence of the CID and determined the desired range of CID concentration to use in the assay (Figure 2a). We then tested candidates from each of the TetR classes as protein receptors and observed that the Y3H system is responsive to the CID with all known TetR classes except for the TetR(C) variant we used (Figure 2b). Given that TetR(B) and TetR(G) showed the best response to the CID and to maintain consistency with the highly studied TetR(B),[37−39] we focused our further experiments on TetR(B).

We show that our assay can differentiate sub-μM concentrations of various tetracyclines (Figure 2c), key for its applicability to differentiate between high and low producer strains. Our Y3H system differentiates doxycycline, tetracycline, and 9-NH_2-minocycline concentrations in the range 2−

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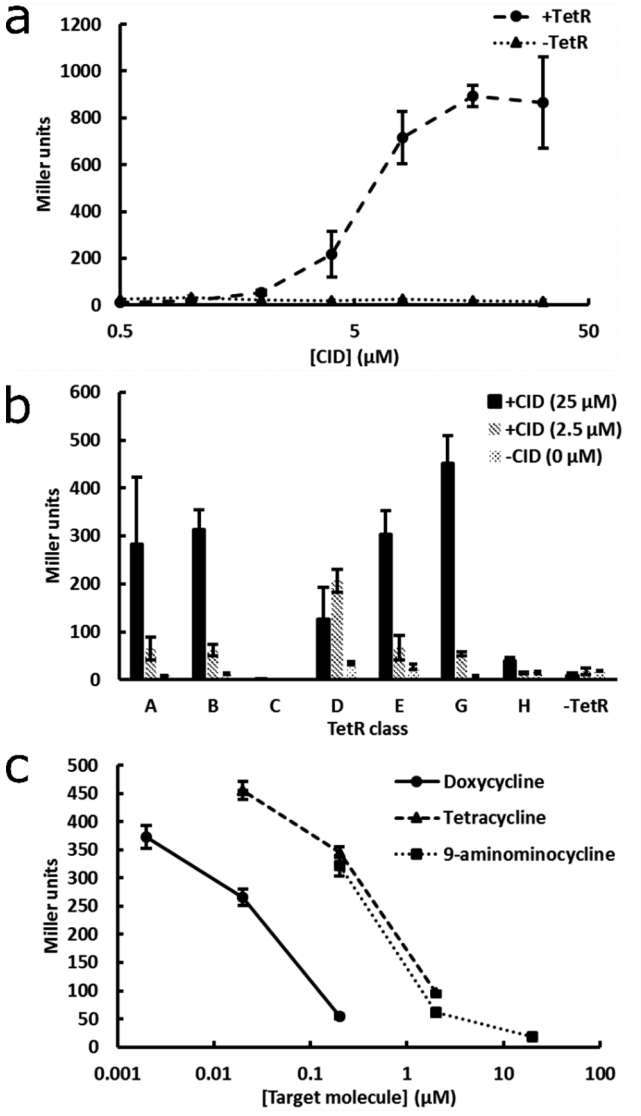

Figure 2. Characterization of the dynamic range of the Y3H assay for tetracyclines in (a) varying concentrations of the Min-Mtx CID, (b) varying TetR classes and (c) varying target molecule structure and concentrations. LacZ was used as a reporter gene. Miller units were calculated as (1000 × OD_{420})/(well volume (mL) × reaction time (min) × OD_{600}).[56] "+TetR" and "−TetR" are strains HL-260−5 and HL-260−7 encoding LexA-TetR(G) and LexA, respectively; Error bars represent the standard error of the mean (SEM) from three biological replicates.

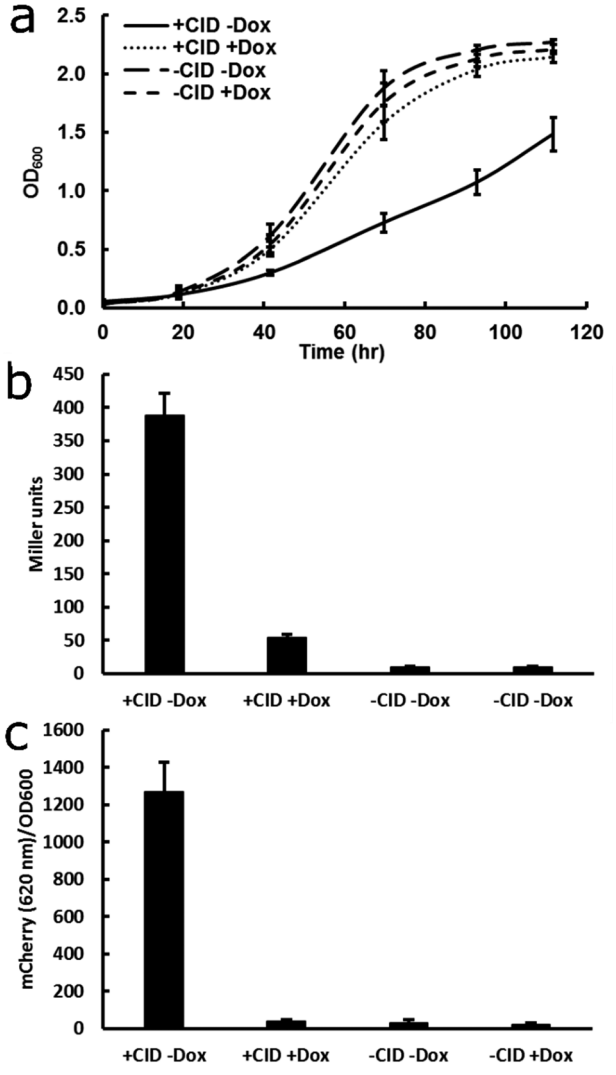

Figure 3. Modularity of the yeast three hybrid assay enables both screening and selecting for biosynthesis of target tetracyclines by enabling (a) a growth assay, (b) a colorimetric assay, and a (c) fluorometric assay. (a) Yeast growth, (b) LacZ transcription, and (c) mCherry transcription are dependent on doxycycline (Dox) in the presence but not in the absence of the CID. CID, Dox concentrations = (a) 25 μM, 5 μM; (b) 25 μM, 0.2 μM; (c) 10 μM, 1 μM. Error bars represent the standard error of the mean (SEM) from three biological replicates. Miller units were calculated as (1000 × OD_{420})/(well volume (mL) × reaction time (min) × OD_{600}).[56] mCherry (620 nm)/OD_{600} was calculated by dividing mCherry fluorescence (620 nm, λ_{ex} = 588 nm) by OD_{600}.

200 nM, 0.02−2 μM, and 0.2−20 μM, respectively. The limit of detection in our Y3H assay is ≤0.2 μM for doxycycline and ≤2 μM for tetracycline and 9-NH_2-minocycline.[32]

After confirming the functionality of the Y3H system for tetracyclines, we proceeded to show that it can report the presence of tetracyclines with a modular output, key for enabling versatile use by various end users. Using a *URA3* reporter gene and growing the yeast in the presence of 5-fluorouracil (5-FOA), we show that yeast growth is doxycycline-dependent in the presence, but not in the absence of the Min-Mtx CID (Figure 3a). We additionally show that the Y3H assay for tetracyclines can be used as a colorimetric (Figure 3b) as well as a fluorometric assay, by employing a LacZ or and mCherry reporter gene, respectively (Figure 3b,c). As expected, while reporter gene activity is dependent on CID and doxycycline in the Y3H strain, such dependence is not detected in a control strain excluding the eDFHR-AD fusion protein (Figure S1).

We demonstrate the applicability of the Y3H assay to metabolic engineering by differentiating between producer and nonproducer strains of TAN-1612 (Figure 4a).[30] For the TAN-1612 producer strain, we used *S. cerevisiae* strain EH-3−54−4, encoding all four genes of the TAN-1612 biosynthetic pathway as previously reported by Tang and co-workers and producing ∼60 μM TAN-1612 (Supplementary Methods in the Supporting Information).[30] The nonproducer control we used, *S. cerevisiae* strain EH-3−54−2, does not encode the

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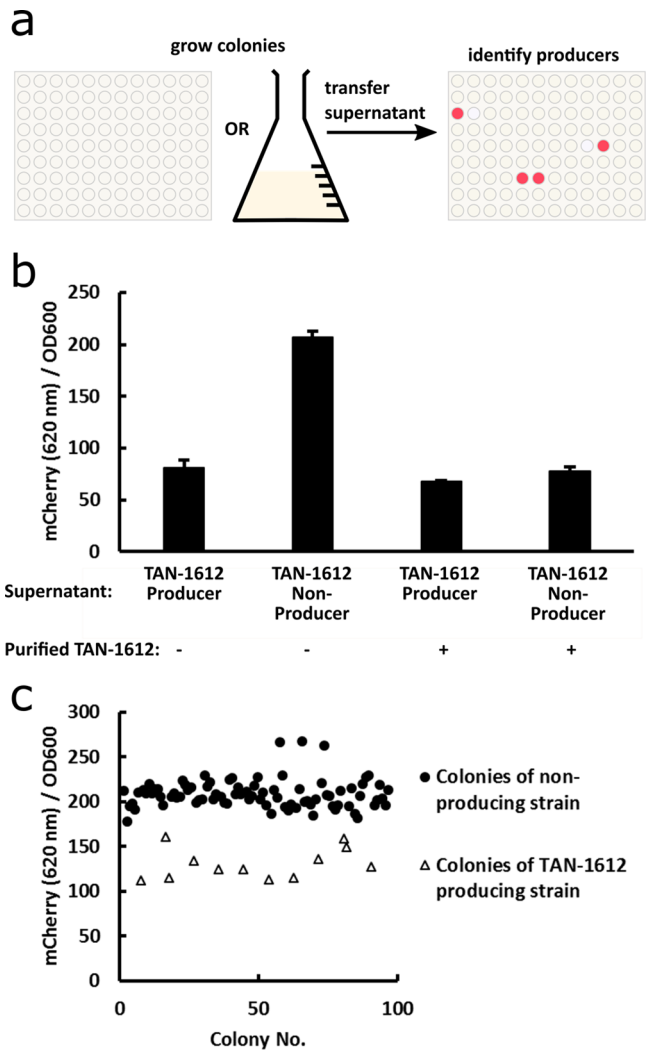

Figure 4. Differentiation of a producer and a nonproducer strain of a target molecule by the Y3H assay for tetracyclines, demonstrating the applicability of the Y3H assay to metabolic engineering. (a) Workflow for screening colonies for production of a target molecule using the Y3H assay. (b) The Y3H assay for tetracyclines differentiates between the supernatants of TAN-1612 producer and TAN-1612 nonproducer cultured in shake flasks. (c) The Y3H assay for tetracyclines differentiates the supernatants of TAN-1612 producer colonies from a nonproducing population cultured in a 96-well plate. The nonproducer strain does not encode AdaA, the polyketide synthase of the TAN-1612 biosynthetic pathway, while the producer strain encodes the complete pathway (Tables S1 and S2). The concentration of TAN-1612 in the Y3H assay well from the supernatant and from the purified sample is ~0.3 and 0.5 μM, respectively (Supplementary Methods in the Supporting Information). mCherry (620 nm)/OD_{600} was calculated by dividing mCherry fluorescence (620 nm, λ_{ex} = 588 nm) by OD_{600}. Error bars represent the standard error of the mean (SEM) from three biological replicates of the Y3H strain and two biological replicates of the TAN-1612 producer/nonproducer.

polyketide synthase AdaA and has no measurable production of TAN-1612 (data not shown).

The Y3H assay for tetracyclines can differentiate between supernatants of producer and nonproducer strains of TAN-1612 (Figure 4b). When purified TAN-1612 is spiked into nonproducer supernatants, the resulting Y3H signal resembles the signal obtained with producer supernatant (Figure 4b). These results agree with our hypothesis that TAN-1612, present in the producer strain supernatant but not in the nonproducer strain supernatant, outcompetes the CID from the LexA-TetR fusion protein.

The Y3H assay for tetracyclines also differentiates TAN-1612 producer colonies from a nonproducing population cultured in a 96-well plate (Figure 4c). The 12 producer colonies gave an average mCherry fluorescence/OD_{600} signal of 132 with a standard deviation of 17, and the 84 nonproducer colonies gave an average mCherry fluorescence/OD_{600} signal of 210 with a standard deviation of 16. Thus, assuming normal distribution there is less than a 2.5% overlap in the Y3H signal from producer and nonproducer colonies.

DISCUSSION

The Y3H assay for tetracyclines is advantageous compared to the state-of-the-art methods for tetracycline detection in terms of throughput, cost, instrumentation requirements, and limit of detection. Being readily performed in 96-well microtiter plates, the Y3H assay throughput is >1000 samples/hour when measured by a standard laboratory plate reader (Infinite-M200). By comparison, the throughput of the previous state-of-the-art HPLC-based methods for tetracycline detection is <10 samples/hour.[33,57] The use of a plate reader to measure the Y3H assay output is also advantageous in terms of cost to state-of-the-art chromatography-based methods such as HPLC and even more so, LC-MS. The price difference is both in the initial investment in instrumentation, as spectrophotometers are cheaper than LC instruments, and in the reagent costs of 96-well plates vs LC-grade solvents. In terms of limit of detection, the Y3H assay has a limit of detection of ≤200 nM for doxycycline (Figure 2c), which is better than the state-of-the-art HPLC limit of detection of ≥4.5 μM for doxycycline.[33,57] The low limit of detection our assay exhibits enables it to differentiate producer from nonproducer strains even in low titers of initial strain development (Figure 4 and Supplementary Methods in the Supporting Information, ~60 μM). Differentiating between production levels in higher titer ballparks in later stages of producer strain optimization would simply require a higher dilution factor of supernatants into the Y3H strain media. Future generations of this Y3H assay might include TetR mutants that are more specific and have a lower detection limit to tetracycline derivatives of interest.

The Y3H assay for metabolic engineering also compares well with our recently published FP assay[58] because of two key differences in setup requirements and output. First, since the Y3H assay does not require the purification of the protein receptor, it is used much more readily to screen for the ultimate protein receptor for the assay. Thus, we show here the first comparison of all TetR classes for binding of a small molecule in an attempt to identify the best TetR variant for differentiating between + and − CID (Figure 2b). Further optimization of a protein receptor for the assay of a specific target molecule is readily enabled in the Y3H assay simply by cloning the protein variants into the same plasmid backbone and transforming into yeast. The versatility in choice of output in the Y3H assay is a key enabling factor for the advantages the Y3H assay has over the FP assay since it makes the assay accessible to a wider range of users. The fluorescent output in the Y3H assay enables measurements with a standard laboratory plate reader, eliminating the requirement for anisotropy capacities (Figure 3c). The LacZ output could

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also be measured by a standard plate reader as well as by the naked eye (Figure 3b).

If the Y3H system and the metabolic pathway for the small molecule of interest are cloned into the same strain, colonies can be assayed for production even more directly, increasing throughput and minimizing handling. The Y3H assay can then enable screening colonies for production of the target molecule directly on an agar plate without the need for further handling of the colonies in microtiter plates. The agar plate screen could be performed with the naked eye or a simple camera by using X-Gal plates and the LacZ reporter or fluorescent camera/microscope by using the mCherry reporter. Finally, the Y3H assay throughput could be vastly increased by selecting for high-producing strains en masse in flask or fermenter culturing using the growth assay or fluorescence assisted cell sorting (FACS). Such en masse screening methods not only greatly enhance the throughput but also minimize the labor, instrumentation, cost, and time required to obtain results. The Y3H assay for tetracyclines can report the presence of a target molecule with any of the above-mentioned outputs (Figure 3), enabling metabolic engineering applications for a range of end users with varying needs and instrumentation availabilities.

While a TetR-*tetO*-based assay could conceivably be developed for the metabolic engineering of tetracycline derivatives,[59] our TetR-based Y3H assay is advantageous for two main reasons. For one, the Y3H assay can potentially be applied to a greater variety of tetracycline derivatives as TetR-binding to the tetracycline suffices and there is no demand for a TetR conformational change. By comparison, signal transduction in the TetR-*tetO* system is dependent on coupling between tetracycline binding and a conformational change in TetR.[26,38] It has been hypothesized that high-affinity binding may be a necessary but not sufficient property for TetR induction in the TetR-*TetO* system.[60] In practice, while it was possible to detect binding to a tetracycline analogue, 4-de(dimethylamino)-anhydrotetracycline, no induction was detected using a TetR-*TetO*-based assay.[37] Thus, the use of TetR in a Y3H system can facilitate the evolvement of TetR mutants with specific binding to tetracycline derivatives of the interest in future generations of the assay. Furthermore, the Y3H system for metabolic engineering can be readily modified to apply for many metabolites of interest beyond tetracyclines, as detailed below. In contrast, the TetR-*TetO* system is much more limited in the scope of molecules it could be applied to, namely, transcriptional regulators.[61]

A Y3H system as described here for the metabolic engineering of tetracycline derivatives can be designed for any target molecule with a receptor. Given T, a desirable target, PR, a protein receptor for T, and T′, a readily derivatizable analogue of T, two new components need to be generated for a Y3H assay to detect biosynthesis of T. The first is a Mtx-T′ CID, and the second is a LexA-PR fusion protein (Figure 1). This approach is especially amenable for the development of assays for molecules of pharmacological interest. The reason is that such molecules often have a known protein target as well as an established derivatization chemistry and SAR that greatly support the design and synthesis of a CID. More broadly, building from our early reports of chemical complementation,[17,62,63] this work advances the enabling technology of the Y3H system for the emerging field of synthetic biology for handling the diversity of engineering multigene pathways and assaying for chemistry not natural to the cell.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00419.

LacZ readout of strain harboring plasmid encoding for LexA-TetR (PBA-8) and strain without the plasmid, a standard curve for TAN-1612 quantification in cultures, supplementary methods, strains and plasmids used in this study, sequences of the various TetR classes within the pMW103 backbone, and sequence of 8LexAOp-minpGal1-mCherry-tCyc1 in the context of pRS416-HL-174-2 (PDF)

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Author Contributions

E.H. designed the Y3H system for tetracyclines. E.H. designed and synthesized the Min-Mtx CID. E.H., H.L., and P.B.A. cloned plasmids and yeast strains. P.B.A. performed Y3H growth assays. E.H. and H.L. performed Y3H LacZ and fluorescence assays. P.B.A. performed TAN-1612 quantification. E.H. and V.W.C. wrote the manuscript. V.W.C. supervised the project.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AD, activation domain; DBD, DNA binding domain; DCM, dichloromethane; DHFR, dihydrofolate reductase; DMF, dimethylformamide; 5-FOA, 5-fluoroorotic acid; HPLC, high-pressure liquid chromatography; LC-MS, liquid chromatography mass spectrometry; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PR, protein receptor; TetR, tetracycline repressor; TFA, trifluoroacetic acid; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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