Counter-selectable marker for bacterial-based interaction trap systems

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*Counter-selectable markers can be used in two-hybrid systems to search libraries for a protein or compound that interferes with a macromolecular interaction or to identify macromolecules from a population that cannot mediate a particular interaction. In this report, we describe the adaptation of the yeast URA3/5-FOA counter-selection system for use in bacterial interaction trap experiments. Two different URA3 reporter systems were developed that allow robust counter-selection: (*i*) a single copy F′ episome reporter and (*ii*) a co-cistronic HIS3-URA3 reporter vector. The HIS3-URA3 reporter can be used for either positive or negative selections in appropriate bacterial strains. These reagents extend the utility of the bacterial two-hybrid system as an alternative to its yeast-based counterpart.*

INTRODUCTION

Many in vivo interaction trap systems have been developed for the identification of protein-protein, protein-DNA, and protein-RNA interactions. These methods typically involve the use of a "bait" (a specific protein, DNA, or RNA sequence) to identify an interacting partner from a library of different "prey." These experiments can take the form of one-hybrid (1,2), two-hybrid (3), or three-hybrid (4) selections and can be performed in yeast (3,5) or bacteria (6–9). Interaction trap systems were originally developed for use in *Saccharomyces cerevisiae*, with interacting partners identified by positive selection. Positive selection is typically achieved by inactivating a gene, such as *HIS3*, required for survival under certain specific growth conditions, and then introducing a reporter system that drives expression of a functional copy of the deleted gene if a desired interaction occurs between the bait and prey (1). For *HIS3*, the stringency of the selection can be manipulated by varying the concentration of 3-amino triazole (3-AT), a competitive inhibitor of HIS3, in the media.

The *URA3* gene (orotidine-5′-phosphate decarboxylase) was originally developed as a counter-selectable marker for yeast (10,11). The endogenous *URA3* gene can be inactivated, and a reporter cassette introduced in which expression of *URA3* is dependent on the presence of a bait/prey interaction. If *URA3* is expressed, the uracil biosynthesis pathway will be functional, and 5-fluoro-orotic acid (5-FOA), which is added to the media, is metabolized into a suicide substrate for the essential thymidylate synthase enzyme. URA3/5-FOA systems have been used successfully in yeast interaction trap systems to counter-select against bait/prey interactions in vivo (5,12). The stringency of this counter-selection can be titrated by varying expression of the *URA3* gene; higher levels of URA3 expression increase the sensitivity of a cell to 5-FOA (12).

pyrF is the *Escherichia coli* homolog of *URA3*, and deletion of *pyrF* can also be complemented by expression of the yeast *URA3* gene (13,14). The URA3/5-FOA system has been used to select against the expression of a functional *URA3* gene in bacteria (14), but it has not been employed in the context of an interaction trap selection system in bacteria. For certain applications, bacteria provide a superior in vivo selection system to yeast (7,15,16). The transformation efficiency of bacteria is approximately 1000 times greater than yeast, which permits the interrogation of much larger libraries. Furthermore, fundamental differences in mechanism between prokaryotic and eukaryotic transcriptional regulation reduce the possibility of interactions between baits or preys of eukaryotic origin and components of the bacterial transcriptional system that could lead to constitutive activation or repression of the reporter gene.

A counter-selection system for bacteria would provide a mechanism for eliminating self-activating baits/preys from libraries (5,17) and would facilitate the identification of mutations that inhibit a protein-protein, protein-DNA, or protein-RNA interaction, thereby permitting the mapping of important functional residues at a recognition interface (5,12). Given the potential utility of a counter-selectable marker in bacteria, we developed a *URA3* reporter system for bacterial-based interaction trap systems (Figure 1). This counter-selectable marker eliminates functional bait-prey interactions with a low breakthrough rate. We also constructed a tandem *HIS3-URA3* reporter system that provides the flexibility of performing positive or negative selections using a single reporter construct.

MATERIALS AND METHODS

ΔpyrF Bacterial Strain Construction

The *pyrF* gene was deleted from two different *E. coli* strains, KJ1C and US0, which bear a preexisting deletion in the *hisB* gene (Reference 7 and J. Hurt, J.K. Joung, and C.O. Pabo, unpublished data). The US0 strain is isogenic to KJ1C, except that it lacks a *Tn10* insertion positioned near the *lac* operon deletion present in both strains. The *pyrF* gene was inactivated in both strains using a λ red recombination system (18). Primers 5′-**GCCCATCA TCAAGAAGGTCTGGTCATGAC GTTAACTGCTTCATCTTCTTCC** GTGTAGGCTGGAGCTGCTTCG-3′ and 5′-**TCATGCACTCCGCTGTAA AGAGGCGTTGATCGCTTTCAGC GTCTGCGCTGGA**CATATGAATAT

CCTCCTTAG-3′ were used to amplify a region of the pKD4 recombination plasmid with about 50 bp of homology to *pyrF* (bolded letters in the primer sequence) at each end. The PCR product was digested with *Dpn*I to remove any plasmid template, gel-purified, and transformed into electrocompetent KJ1C and US0 cells containing a λ red helper plasmid (pKD46). The transformants were recovered in SOB media with 10 mM L-arabinose and screened for recombination on kanamycin plates. Candidate insertions in the *pyrF* gene were confirmed by PCR using primers that amplify *pyrF* (5′-AGGGGAGAAATCGCAACTGT-3′, 5′-CTTATCGCCCTGGATTTCAA-3′). The kanamycin gene was removed from positive clones by FLP-mediated recombination by introducing plasmid pCP20. The scars remaining from the recombination process in the KJ1C and US0 strains were confirmed by sequencing the PCR-amplified region of the genome. The F′ episome from XL1-Blue (Stratagene, La Jolla, CA, USA), which contains *lacI*^{q} was introduced into US0*ΔhisBΔpyrF* through conjugation followed by selection on minimal medium containing 0.1% histidine, 0.1 mM uracil, 10 µg/mL tetracycline, and 2.5 mM 5-FOA.

Counter-Selection Medium

Yeast extract minimal medium (YM) is composed of M9 minimal medium supplemented with 10 µM $ZnSO_4$, 100 µM $CaCl_2$, 1 mM $MgSO_4$, 10 µg/mL thiamine, 25 µg/mL kanamycin, 30 µg/mL chloramphenicol, 200 µM uracil, 0.1% histidine, 0.01% yeast extract, and a mixture of 17 amino acids (excluding histidine, methionine, and cysteine) (19). 5-FOA (2.5 and 0.2 mM) was used for the counter-selections of pH3U3 and F′ episome reporter, respectively. The composition of NM media has been previously described (19). For selective plates, 1.8% agar was added to the medium mixture.

Plasmids and Strains Used in the Bacterial Counter-Selection System

The bacterial counter-selection system is derived from a previously described bacterial two-hybrid system (7). Zif268 and ZnFp53 DNA binding domains (DBDs) (20,21) were expressed as direct fusions to the α subunit of RNA polymerase (α-Zif268 or α-ZnFp53) using a modified pACL-αGal4 vector (7), which contains a p15A origin of replication and chloramphenicol resistance marker. DNA encoding a short linker and each DNA binding domain was substituted for Gal4 between the *Not*I and *Avr*II sites. An additional ampicillin resistance marker was introduced into the α-ZnFp53 expression plasmid to distinguish it from the α-Zif268 plasmid.

Two types of *URA3* reporters were developed for the counter-selection system: (*i*) the *URA3* F′ episome reporter and (*ii*) the pH3U3 reporter. The *URA3* F′ episome reporter is architecturally identical to the previously reported *HIS3* (7) and *lacZ* (16) F′ episome reporters. The p1566-*URA3-aadA* plasmid for creating the F′ episome reporter was constructed by substituting *URA3* from *S. cerevisiae* for *HIS3* between the *Bam*HI and *Xba*I restriction sites in p1566-*HIS3-aadA*, which has a p15A origin of replication and kanamycin resistance marker. DNA binding sequences for Zif268 or ZnFp53 (Zif268: 5′-GCGTGGGCG-3′; ZnF53: 5′-GGGACACGT-3′) (20,21) were introduced into p1566-*URA3-aadA* centered 62 bp upstream of the transcription start site of the weak *lac* promoter (7). The *URA3* reporter cassettes were integrated into the F′ episome of the CSH100 strain via recombination and then introduced into the KJ1C*ΔhisBΔpyrF* strains by conjugation to make the *URA3* F′ episome reporter strains (7,16,22). An alternate plasmid-based reporter (pH3U3), which contains a *HIS3-URA3* cistron was constructed that allows both positive and negative selections (23). Reporters containing the Zif268 or ZnFp53 sites in pH3U3 were generated, and the selections using these vectors were performed in the US0*ΔhisBΔpyrF* strain containing the F′ episome from XL1-Blue.

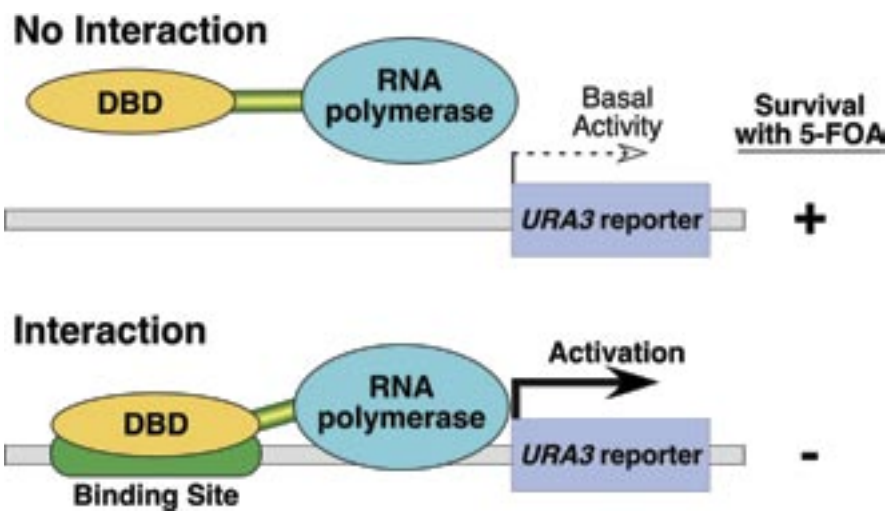

Figure 1. Overview of the bacterial-based URA3 counter-selection system. Survival of US0*ΔhisBΔpyrF* transformants in the presence of 5-fluoro-orotic acid (5-FOA) depends on the absence of an interaction between the DNA binding domain (DBD) and a DNA sequence upstream of the *URA3* reporter in a one-hybrid configuration (top). If the DBD binds upstream of the promoter, it will recruit RNA polymerase through a direct fusion to an RNA polymerase subunit and stimulate *URA3* expression. In principle, the stronger the interaction between the DBD and a sequence upstream of the promoter, the less tolerant an individual cell will be to 5-FOA due to increased expression of *URA3* (12).

URA3/5-FOA Counter-Selection Procedure

Electrocompetent cells were prepared of KJ1C*ΔhisBΔpyrF* strains bearing different F′ episome *URA3* reporters and of US0*ΔhisBΔpyrF* strains bearing different pH3U3 reporters. These competent cells were transformed with the α-Zif268 or α-ZnFp53 expression vector and recovered in 1 mL SOC for 1 h at 37°C. Following recovery, these cells were pelleted and resuspended in 1 mL YM

containing 25 µg/mL kanamycin and 30 µg/mL chloramphenicol and grown for 1 h at 37°C. The cells were pelleted and washed three times with 1 mL sterile water and then resuspended in 1 mL YM for counter-selection on YM plates containing 25 µg/mL kanamycin, 30 µg/mL chloramphenicol, 10 µM isopropyl-β-D-thiogalactopyranoside (IPTG), and either 0.2 or 2.5 mM 5-FOA. Plates were incubated at 37°C overnight.

RESULTS AND DISCUSSION

Creation of *ΔpyrF* Bacterial Strains

pyrF, which is the bacterial homolog of *URA3*, was inactivated in two *ΔhisB E. coli* strains, KJ1C and US0, which were previously developed for HIS3/3-AT-based positive selection (7). The *pyrF* gene was interrupted using the λ red homologous recombination protocol described by Datsenko and Wanner (18). PCR analysis of the KJ1C and US0 isolates constructed using this procedure confirmed that the majority of the *pyrF* gene was deleted from the genome (data not shown). These new *ΔhisBΔpyrF* strains are resistant to 5-FOA and require uracil and histidine for growth on NM media (Figure 2A).

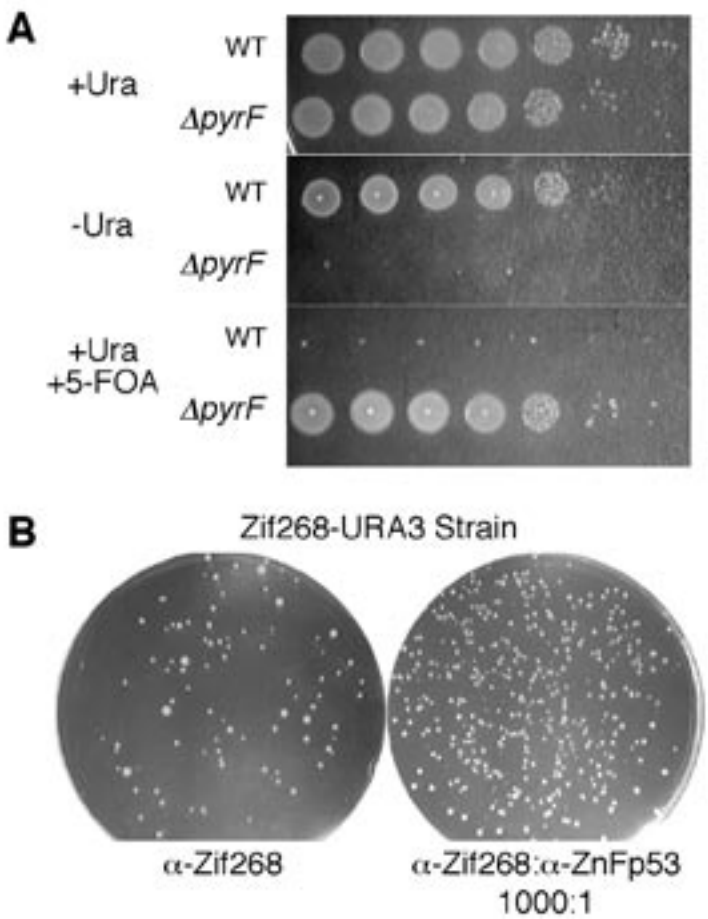

Figure 2. Deletion of the *pyrF* gene in the US0 bacterial strain inactivates the uracil biosynthesis pathway, permitting *URA3* to be used as a counter-selectable marker. (A) Tenfold serial dilutions of cells from one of the US0*ΔhisBΔpyrF* strains and the parent US0*ΔhisB* strain (WT) were spotted in 5-µL drops on NM minimal plates with histidine and either containing uracil, lacking uracil, or containing uracil and 2.5 mM 5-fluoro-orotic acid (5-FOA). The *ΔpyrF* strain requires uracil in the media to survive and tolerates the presence of 5-FOA. (B) Counter-selection using the Zif268-*URA3* F′ episome reporter strain. Left plate, approximately 5×10^5 cells of the Zif268-*URA3* F′ episome reporter strain containing the α-Zif268 expression vector were plated on one 150 × 15 mm 0.2 mM 5-FOA plate, and 80 colonies survived. Right plate, approximately 7×10^5 cells of the Zif268-*URA3* F′ episome reporter strain containing a mixture of the α-Zif268 and α-ZnFp53 expression vectors at a ratio of 10^3 to 1 were plated on one 150 × 15 mm 0.2 mM 5-FOA plate, and about 450 colonies survived. The α-ZnFp53 expression vector contains an extra antibiotic marker (amp^R) that allows cells containing this vector to be distinguished from cells containing the α-Zif268 expression vector. Based on the presence of this extra marker, approximately 85% of surviving colonies contained the α-ZnFp53 expression vector. The 5-FOA counter-selection using the F′ episome *URA3* reporter was effective at cell densities as high as 10^7 cells on a 150 × 15 mm round plate.

Evaluation of the URA3/5-FOA Bacterial Counter-Selection System

The effectiveness of URA3/5-FOA counter-selection system in bacteria was evaluated using a single copy reporter integrated into the F′ episome. This arrangement is homologous to the *HIS3* and *lacZ* reporters previously developed for the bacterial two-hybrid system (7,16). Cells containing the *URA3* reporter at its basal level of activity can tolerate media containing 0.2 mM 5-FOA. Two *URA3* reporters were constructed (Zif268-*URA3* and ZnFp53-*URA3*) that contain a binding site for the corresponding zinc finger proteins, Zif268 or ZnFp53 (20,21), upstream of the promoter (Figure 1). The Zif268-*URA3* and ZnFp53-*URA3* F′ episome reporters should be selectively activated by the Zif268 and ZnFp53 proteins, respectively, when these proteins are expressed as fusions to the α-subunit of RNA polymerase (6,24). Electrocompetent cells containing each *URA3* reporter were transformed with expression vectors for α-Zif268 or α-ZnFp53. Following recovery, cells containing the four different combinations of reporters and DBDs were challenged to survive on minimal medium plates containing 0.2 mM 5-FOA. Cells containing matched reporters and DBDs (ZnFp53-*URA3*/α-ZnFp53 or Zif268-*URA3*/α-Zif268) displayed a significantly reduced survival rate (about 1 in 10^4 cells), whereas cells containing mismatched reporters and DBDs displayed nearly complete survival (Figures 2B and 3). A mixed population of expression vectors (where the α-Zif268/α-ZnFp53 ratio is 10^3 to 1) was used to test the ability of the URA3/5-FOA counter-selection to isolate a desired subpopulation through selection. This mixture was transformed into the selection strain containing the Zif268-*URA3* reporter. Approximately 85% of the

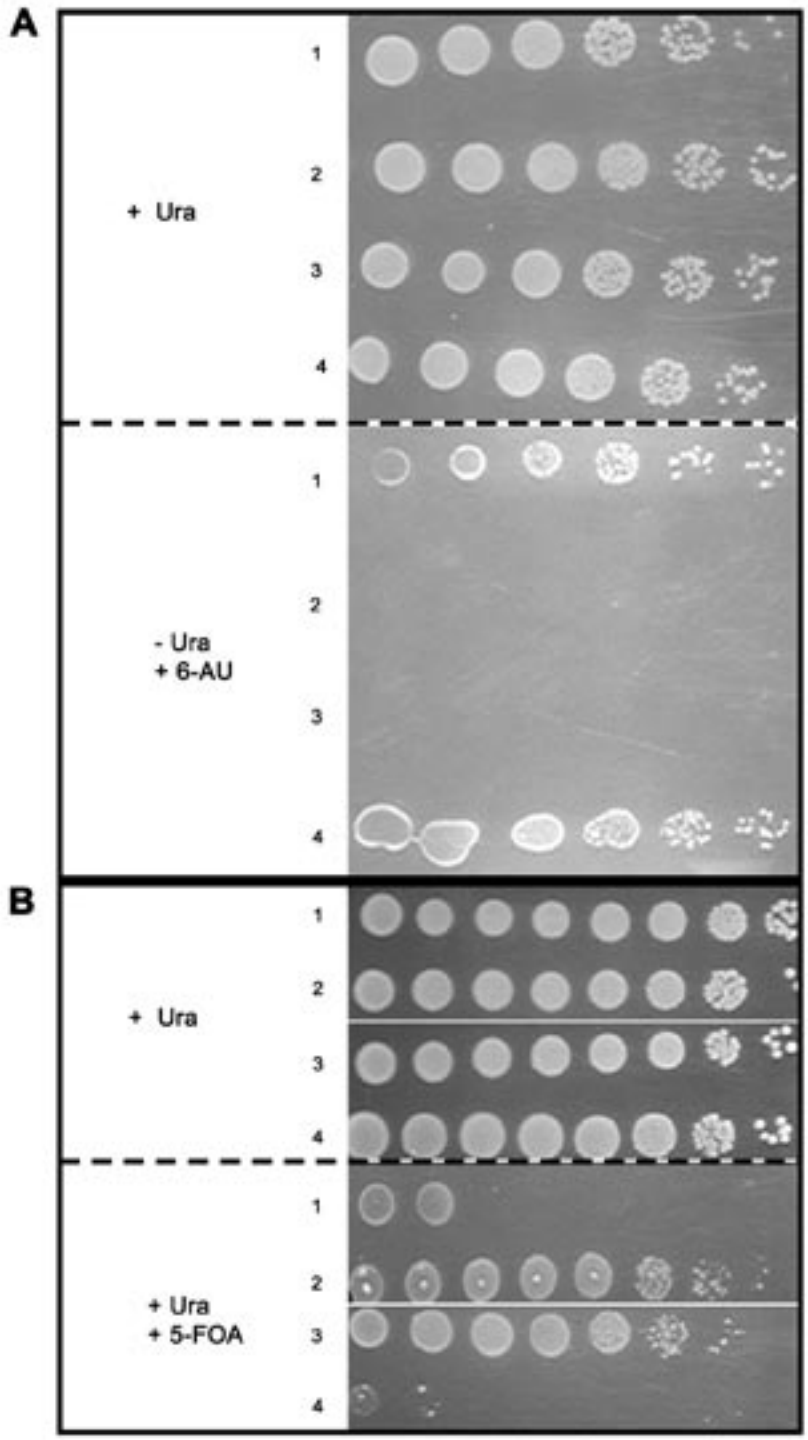

Figure 3. Activation of the *URA3* F′ episome reporter allows survival in the presence of 6-azauracil (6-AU) and eliminates cells in the presence of 5-fluoro-orotic acid (5-FOA). Fivefold serial dilutions of KJ1C*ΔhisBΔpyrF* cells containing each of four combinations of F′ episome reporters and DNA binding domains (DBDs) were spotted in 5-µL drops on plates with or without selective media in the following order: row 1, the Zif268-*URA3*/α-Zif268; row 2, the Zif268-*URA3*/α-ZnFp53; row 3, the ZnFp53-*URA3*/α-Zif268; and row 4, ZnFp53-*URA3*/α-ZnFp53. (A) Cells were plated on NM media containing either uracil or 6-AU, and colonies were observed on the 6-AU plate only in the matched reporter/DBD sets (rows 1 and 4), although some toxicity was observed even in these sets at high cell densities. (B) Cells were plated on either yeast extract tryptone medium (2× YT) or yeast extract minimal medium (YM) containing 0.2 mM 5-FOA. In this case, nearly complete survival is observed for the mismatched sets (rows 2 and 3), whereas effective elimination of the matched sets is observed (rows 1 and 4).

cells that survived the counter-selection contained the α-ZnFp53 construct (Figure 2B). The increased activity of the *URA3* F′ episome reporter in the matched reporter/DBD combinations was confirmed by titrating cells on uracil-deficient media containing 6-azauracil (6-AU), a compound that is metabolized into a competitive inhibitor of the URA3 enzyme (25,26). As expected, under these conditions, only matched reporters and DBDs (ZnFp53-*URA3*/α-ZnFp53 or Zif268-*URA3*/α-Zif268) produced colonies (Figure 3).

A new bacterial counter-selection reporter vector was constructed to decrease the breakthrough rate of 5-FOA counter-selections and to allow positive (HIS3) or negative (URA3) selections to be performed with the same construct. The pH3U3 reporter plasmid contains a synthetic *HIS3-URA3* cistron expressed from the weak *lac* promoter (23). Combinations of pH3U3 reporter plasmids (containing either Zif268 or ZnFp53 binding sites) and expression plasmids (α-Zif268 or α-ZnFp53) were introduced into the US0*ΔhisBΔpyrF* strain to examine the effectiveness of 5-FOA counter-selection. Following recovery, the four different combinations of transformants were titrated on 2.5 mM 5-FOA YM plates. A higher efficiency of killing was observed for the URA3/5-FOA counter-selection when using the pH3U3 reporter than when using the F′ episome *URA3* reporter (Figure 4A). Cells containing matched reporters and DBDs (Zif268-pH3U3/α-Zif268 or ZnFp53-pH3U3/α-ZnFp53) displayed a lower breakthrough rate (about 1 in 10^5 cells), while cells containing mismatched reporters and DBDs (Zif268-pH3U3/α-ZnFp53 or ZnFp53-pH3U3/α-Zif268) were fully tolerant of these conditions. The opposite result is obtained when a HIS3/3-AT selection is performed; only cells that contain matched reporters and DBDs survive in the presence of 2 mM 3-AT (data not shown).

A mixed population of expression vectors (where α-Zif268/α-ZnFp53 ratio is 1 to 10^3) was used to test the ability of the URA3/5-FOA counter-selection to isolate a desired subpopulation through selection. This

mixture was transformed into the US0*ΔhisBΔpyrF* strain containing the ZnFp53-pH3U3 reporter, and counter-selections were performed as described above. Only approximately 1 in 10^3 cells survived the selection, which is consistent with the number of Zif268 clones in the population. A similar result was achieved when the ratio of the two vectors was reversed, and the mixture was transformed into the US0*ΔhisBΔpyrF* strain containing the Zif268-pH3U3 reporter. Expression plasmids from nine of the surviving colonies of each selection were prepared and tested by analytical restriction enzyme digestion to identify the transcription factor expressed in each clone (Figure 4B). All nine clones selected using the ZnFp53-pH3U3 reporter contained the Zif268 transcription factor, and all nine clones selected using the Zif268-pH3U3 reporter contained the ZnFp53 transcription factor. As with the F′ episome reporter, approximately 10^7 cells can be challenged on a single selection plate without increased rates of breakthrough.

In summary, we have developed a counter-selectable marker for the bacterial interaction trap system that could be used to search libraries for a protein or compound that interferes with an interaction or to isolate a macromolecule that does not mediate a certain protein-protein, protein-DNA, or protein-RNA interaction. The *URA3* reporter can also be used to remove self-activating clones from a population (23). The URA3/5-FOA counter-selection system can be used in conjunction with an F′ episome reporter or as a linked *HIS3-URA3* reporter vector. The *HIS3-URA3* reporter provides the advantage that it can also be used for HIS3/3-AT-based positive selections using the *ΔhisBΔpyrF* strains described herein. These reagents continue to extend the utility of the bacterial interaction trap system as a powerful alternative to its yeast-based counterpart.

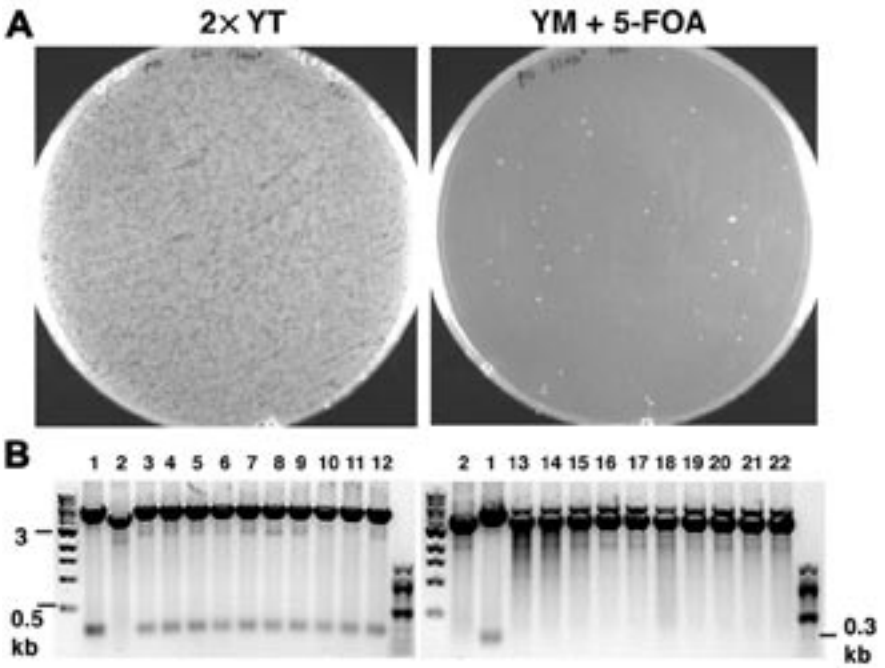

Figure 4. Counter-selection using the pH3U3 reporter system. (A) Counter-selection efficiency of 5-fluoro-orotic acid (5-FOA) using a pH3U3 reporter. Cells (1.3×10^6) containing ZnFp53 expression vector and pH3U3 reporter with ZnFp53 binding site were plated on either a yeast extract tryptone medium (2× YT) plate (no counter-selection) or a yeast extract minimal medium (YM) plate with 2.5 mM 5-FOA. A lawn of cells was produced on the 2× YT plate, while only 20 colonies were apparent on the 5-FOA plate. (B) Selection of a desired expression vector from a mixed population via URA3/5-FOA counter-selection. Cells containing Zif268-pH3U3 reporter were transformed with a mixture of α-Zif268/α-ZnFp53 expression vectors at a ratio of 10^3 to 1. Approximately 10^5 of these cells were challenged to grow on YM with 2.5 mM 5-FOA. Fewer than 100 colonies survived the counter-selection. Plasmid DNA from nine colonies was isolated and analyzed by restriction enzyme digestion (*Not*I). All nine clones contained the α-ZnFp53 vector (lanes 3–12) as evidenced by the presence of the expected 4700- and 250-bp fragments. Lanes 1 and 2 are positive controls of α-ZnFp53 and α-Zif268 vector, respectively, cut by *Not*I. The corresponding experiment was performed with the ZnFp53-pH3U3 reporter, where the ratio of the mixture of α-Zif268/α-ZnFp53 expression vectors was inverted. Plasmid DNA from nine colonies was isolated and analyzed by restriction enzyme digestion (*Not*I). All nine clones contained the α-Zif268 vector as evidenced by the presence of the expected 3900-bp fragment (lanes 13–22). The extra bands visible in lanes 13–22 are from the pH3U3 reporter vector. In each gel, the left unmarked lane is the 1-kb DNA ladder from New England Biolabs (Ipswich, MA, USA), and the right unmarked lane is the 100-bp DNA ladder (also from New England Biolabs).

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COMPETING INTERESTS STATEMENT

X.M., J.K.J., and S.A.W. declare that they have a patent application pending on related technology. All other authors declare no competing interests.

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