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Two-Hybrid System for Characterization of Protein-Protein Interactions in *E. coli*

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ABSTRACT

The yeast two-hybrid system has been used to characterize many protein-protein interactions. A two-hybrid system for E. coli *was constructed in which one hybrid protein bound to a specific DNA site recruits another to an adjacent DNA binding site. The first hybrid comprises a test protein, the bait, fused to a chimeric protein containing the 434 repressor DNA binding domain. In the second hybrid, a second test protein, the prey, is fused downstream of a chimeric protein with the DNA binding specificity of the l repressor. Reporters were designed to express* cat *and* lacZ *under the control of a low-affinity l operator. At low expression levels, l repressor hybrids weakly repress the reporter genes. A high-affinity operator recognized by 434 repressor was placed nearby, in a position that does not yield repression by 434 repressor alone. If the test proteins interact, the 434 hybrid bound to the 434 operator stabilizes the binding of the l repressor hybrid to the l operator, causing increased repression of the reporter genes. Reconstruction experiments with the fos and jun leucine zippers detected protein-protein interactions between either homodimeric or heterodimeric leucine zippers.*

Table 1. Strains and Plasmids

Strain	Genotype	Comments
AG1688	MC1061 F′128 lacI^q, lacZ::Tn5	Reference 15
JH729	AG1688(λLS100)	this work
Reporter phage	Relevant features	
λLS100	O_{434}-8bp-O_λ(weak), P_S-*cat-lac*	
Plasmids		
pLS3	Plac-434R-GCN4(IINI)	Amp^R, ori pBR322, M13
pLS14	pLS3(Fos)	
pLS79	pLS3(Jun)	
pLS82	pLS3(Fos mutant)	
pLS13	P7107-λcI-GCN4(INII)	Tet^R, ori pACYC184
pLS16	pLS13(Jun)	
pLS68	O_{434}-8bp-O_λ(weak), P_S-*cat*	Amp^R, ori pBR322

INTRODUCTION

Microbial systems provide powerful genetic tools for the analysis of protein function. Their rapid growth allows assays to be comparatively fast, and their growth to high titers allows selection of mutants that alter structure or function. In addition, suppressor analysis has often provided clues about protein-protein interactions. Unfortunately, most proteins have no obvious phenotype that can be correlated with their self-assembly in a foreign host. Gene fusion methods including yeast two-hybrid systems (2–4,6), phage display (1,25) and bacterial repressor fusions (16) can provide phenotypic assays for function, allowing the power of genetic methods to be applied to general problems in protein-protein interaction.

In yeast two-hybrid systems, one interaction partner—the bait—is fused to a DNA binding protein that lacks an activation domain. The other partner—the prey—is fused to a transcriptional activation domain that lacks DNA binding activity. Interaction between the bait and the prey recruits the activator to the site recognized by the DNA binding protein, allowing transcription of a reporter gene.

Protein-protein interactions have also been studied in *E. coli* by replacing dimerization domains in bacterial or phage transcription factors (7,8,13,17, 18,28). Methods to study heterodimers based on the coassembly of two monomeric hybrid proteins (10) or negative dominance (7,9,19,23,32) require information about whether or not the test proteins form homodimers. Here, we describe a modification of the λ repressor fusion system that can detect a protein-protein interactions involving either homodimeric and heterodimeric protein domains.

MATERIALS AND METHODS

Strains, Plasmids and Microbiological Methods

The strains, phage and plasmids used in this study are listed in Table 1. Plasmids for the construction of reporters and hybrid proteins were constructed by standard molecular biology methods, and the structures of relevant portions were confirmed by DNA sequencing. The λ-transducing phages carrying

reporters were constructed by homologous recombination with plasmids as described previously (33) and used to lysogenize AG1688. Single lysogens were identified by β-galactosidase assays of the unregulated levels of *lacZ* expression and confirmed by PCR by the method of Powell et al. (27).

The reporter operon is carried on specialized λ imm21-transducing phages and are used as single-copy chromosomal insertions at attλ in the *E. coli* chromosome. λLS100 contains a high-affinity 434 operator spaced 8 bp upstream from a low-affinity λ operator, O_S2. λLS100 was constructed by homologous recombination as described by Zeng and Hu (33), using plasmid pLS68, where a synthetic 434 operator shown in Figure 1 replaced the λ operator in pXZ660.

The bait plasmid, pLS3, is a derivative of pZ150 (31) used to construct fusion proteins to the 434 repressor DNA binding domain. pLS3 contains sequences corresponding to residues 1–79 of the 434 repressor and residues 117–132 from the interdomain linker of λcI repressor followed by the mutant GCN4(IINI) leucine zipper, which contains I, I, N and I at residues 9, 16, 23 and 30 of the leucine zipper (32). Sequences encoding the test baits are inserted between *Mlu*I and *Bam*HI cloning sites at the C-terminal end of the hybrid protein (Figure 2). These sites and an additional *Mun*I site were designed to accept inserts from the yeast two-hybrid vectors from the Elledge (3) and Brent (6) laboratories. We refer to bait hybrid proteins as 434-IINI-X, where X is the specific bait being tested. 434-IINI-X is expressed from the *lacUV5* promoter. pLS3 confers ampicillin resistance and contains origins of replication from pBR322 and M13.

The compatible prey plasmid, pLS13, contains sequences corresponding to residues 1–132 of λcI repressor followed by the mutant GCN4(INII) leucine zipper, which contains I, N, I and I at residues 9, 16, 23 and 30 of the leucine zipper (32), followed by the same cloning sites as pLS3. The difference in the pattern of buried asparagines at these positions precludes heterodimerization of the two-hybrid proteins through their leucine zippers. The prey hybrid protein, referred to hereafter as cI-INII-Y, where Y is the fused prey protein, is expressed from the low-level constitutive promoter P7107 (32) to prevent repression by the cI DNA binding domain in the absence of a protein-protein interaction with 434-IINI-X. pLS13 confers tetracycline resistance and contains an origin of replication from pACYC184.

Plasmids were introduced into the indicated *E. coli* strains by electroporation or by M13-mediated transduction for plasmids carrying M13 origins (30).

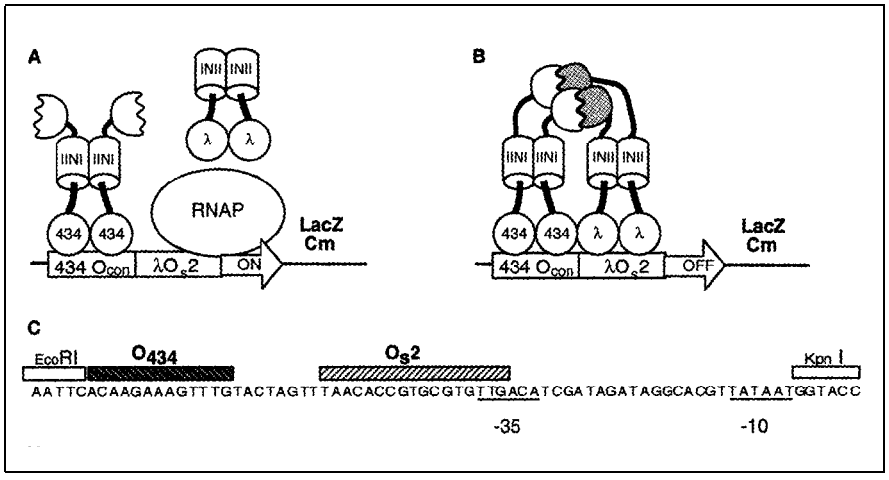

Figure 1. Rationale for the assay. Expression of *cat* and *lacZ* reporter genes is driven by the promoter indicated by arrows in panels A and B and by -35 and -10 labels in panel C. (A) Binding of a 434-INII fusion protein to the upstream 434 operator is not sufficient to repress transcription and has no effect on the binding of a coexpressed λcI-IINI fusion protein to a low-affinity λ operator in the absence of interacting protein domains fused at their C-terminal ends. (B) In the presence of fused domains that can interact, the presence of the 434 fusion recruits the λ fusion to its operator, leading to decreased reporter gene expression.

β-Galactosidase Assays

β-galactosidase assays were performed on log phase cultures grown in LB broth. Similar results were obtained with cells grown in M9 Glucose minimal medium (24) supplemented with casamino acids (data not shown). The assays were performed by the SDS-chloroform method described by Miller (24). Relative β-galactosidase activity is the ratio of the activity measured in each reporter strain and the value obtained on the same day for the same λ cI-IINI prey coexpressed with the control 434-INII fusion protein with no bait. Each value represents the average of duplicate samples run on two strains of identical genotype. All of the strains were assayed on at least two different days, and the error bars represent the SD of the normalized values from independent measurements.

RESULTS

Figure 1 shows the general approach based on a genetic assay for the formation of homotetramers (5,33). Reporter operons were constructed with a weakly binding λ operator, O_S2, which overlaps a synthetic promoter, P_S. Occupancy of O_S2 represses P_S; however, at an appropriate concentration of λ repressor, O_S2 is only partly occupied. A consensus 434 operator is placed upstream of the promoter, where bound 434 repressor is unable to repress transcription from P_S. In the complete system, the bait is fused to the C-terminal end of a 434 repressor-leucine zipper fusion protein. The prey is fused to a λ repressor leucine zipper fusion protein. The dimerization specificities of the leucine zippers in the bait and prey fusions prevent them from forming heterodimers. Interaction between the bait and the prey stabilizes binding of the λ repressor fusion protein to the λ operator and leads to the repression of P_S.

To test whether this system would detect interactions using either homodimeric or heterodimeric leucine zipper motifs as baits, we constructed plasmids expressing 434-IINI-Jun, 434-IINI-Fos or 434-IINI-Fos*, in which the third, fourth and fifth leucines of the zipper motif are changed to valine,

alanine and valine, respectively (29). Jun forms homodimers and heterodimerizes with Fos. The mutation in Fos* is known to abolish the interaction with Jun. Plasmids expressing λ-Jun were used as the test prey. Cassettes encoding the Fos and Jun leucine zippers were obtained by PCR amplification using pYK34 (Fos) and pYK1 (Jun) as templates (21) and an upstream PCR primer that introduces an in-frame *Mlu*I site; the downstream *Bam*HI site is in the amplified segment of the template. The Fos* leucine zipper cassette was constructed from oligonucleotides by mutual primed synthesis. The three cassettes were cloned between the *Mlu*I and *Bam*HI sites of pLS3, and the Jun cassette was cloned into the same sites in pLS13. The plasmids were introduced in pairs into AG1688 lysogenized by λLS100.

Figure 3 shows the results of the reconstruction experiment. β-galactosidase activity is normalized to the value for coexpression of cI-INII fused to each prey in the presence of 434-IINI with no bait. Note that this normalization is important because different hybrid proteins may have different intrinsic repressor activities because of factors including differences in proteolytic turnover rates, steric differences in the fusion proteins and fortuitous cloning of sequences able to activate transcription of the reporter. Repression by the cI-IINI-Jun hybrid is enhanced by 434-IINI-Jun or 434-INII-Fos leucine zippers, but not by 434-INII-Fos*. None of the bait fusions reproducibly increased in repression by the control cI-INII protein. These results demonstrate that cooperative binding of two-hybrid proteins can be used to detect protein-protein interactions in *E. coli*, independent of whether the bait is monomeric or dimeric. Similar results were obtained when the separation between the operators was increased by 5 bp (data not shown), indicating that the fusion proteins are flexible enough to reach both sides of the DNA helix.

DISCUSSION

Two-hybrid methods have shown that protein-protein interactions from a variety of systems can be reconstituted in the environment of the yeast nucleus. Yeast two-hybrid methods have provided a wealth of information about interacting proteins and are now being used to examine networks of how all of the proteins in a proteome interact. Here, we have described a set of vectors that should allow similar genetic assays in *E. coli*. Two-hybrid experiments could be more efficiently done in *E. coli* because it grows faster than yeast and can be transformed with higher efficiency (14).

Recently, three other bacterial two-hybrid systems based on DNA binding proteins have been described based on (*i*) formation of heterodimeric forms of LexA with asymmetric sequence specificities (10), (*ii*) DNA loop formation (22) and (*iii*) recruitment of a transcriptional activation domain (11,12). In addition, other bacterial two-hybrid systems have been developed based on

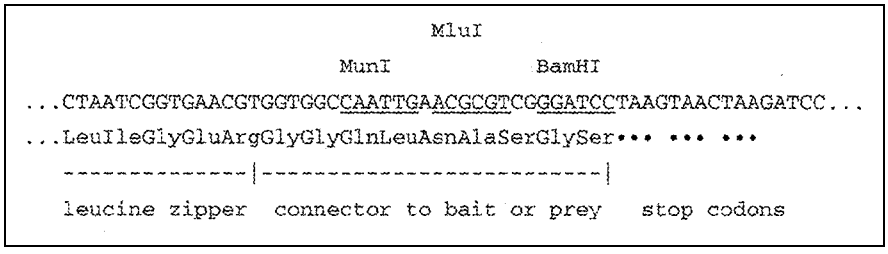

Figure 2. Cloning sites in two-hybrid plasmids pLS3 and pLS13. Fusions can be constructed by inserting foreign protein domains into the *Mun*I, *Mlu*I or *Bam*HI sites in this linker, all of which are unique in both plasmids.

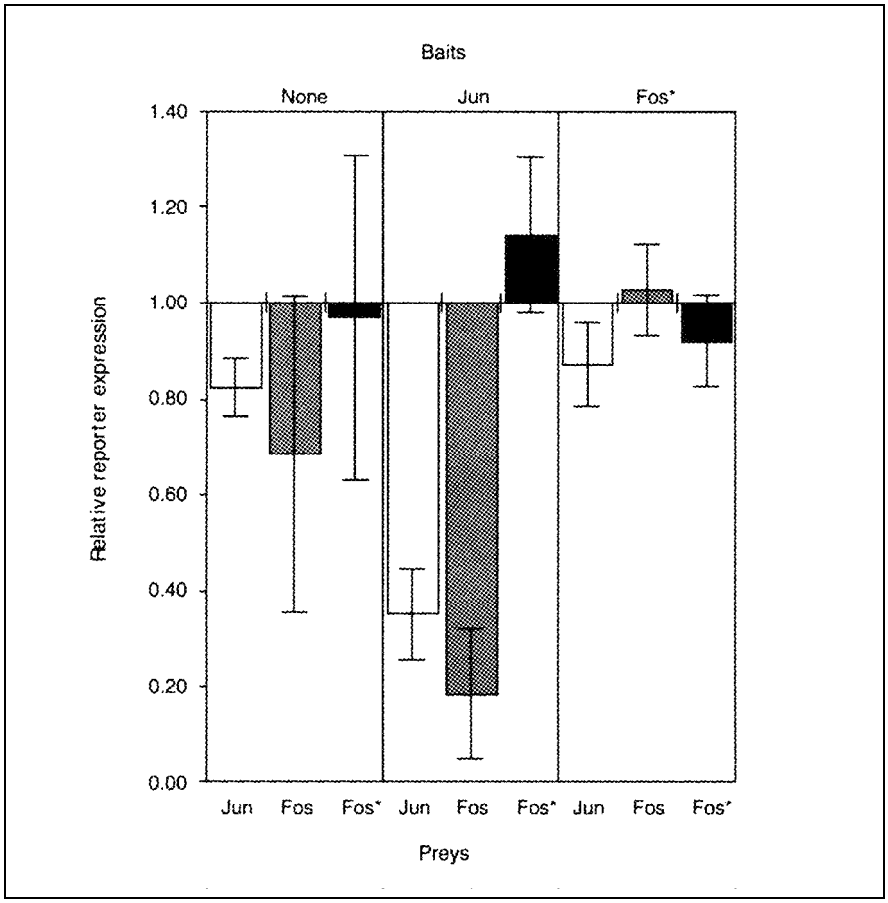

Figure 3. Interaction between bait and prey increases repression. The baits used in each panel are shown above, and the preys are indicated below the graph. Relative β-galactosidase activity was determined as described in Materials and Methods. Absolute β-galactosidase activities varied from day to day with control strains lacking any repressor fusion protein (not shown) giving about 2000 Miller units of activity.

reconstitution of enzyme activity from fragments (20,26). Each of these systems is relatively new, and the relative strengths and weaknesses of each should be revealed with use. The system described here uses much lower protein expression levels than the looping and recruitment systems. This may allow assays with proteins and domains that are toxic to *E. coli* when overexpressed. On the other hand, the signal-to-noise ratio of our system is substantially less than is observed with several other bacterial systems.

We have not yet attempted to use this system to screen cDNA or genomic DNA libraries. Although this should be possible in principle, both of the genetic markers in the reporters we used to demonstrate proof of principle here are selectable for increased expression, while a positive interaction leads to decreased expression. Thus, the current system is better suited to select for loss of interaction (e.g., in screening for drug leads that abolish a known interaction) than for the identification of new interactions among members of a library. In addition, the signal-to-noise level of the system is probably too low to allow meaningful cDNA or genomic library screens with the current generation of reporters. Further work to optimize the reporter constructs might be useful.

All interaction trap methods are subject to the possibility of artifactual false positives and false negatives. These issues have been explored at length for the yeast two-hybrid systems. As tools for examining the interaction of proteins of eukaryotic origin, bacterial systems will benefit from the absence of bridging interactions and the elimination of nuclear localization as a factor in whether or not a signal is detected. However, other factors may lead to the failure of any bacterial system to detect a true positive that is observable in the yeast system. All systems based on phenotypes in the presence of chimeric proteins are constrained by factors including the geometry of the fusion proteins, their expression levels, localization and intracellular turnover. As with any genetic method, negative results from our two-hybrid system will be difficult to interpret, and positives should be used as indicators for further study, rather than as definitive results. Nevertheless, we expect that our approach should be complementary to the other bacterial and yeast two-hybrid systems and other methods to study protein-protein interactions.

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