21 Using λ Repressor Fusions to Isolate and Characterize Self-assembling Domains

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INTRODUCTION

Although there are a wide variety of methods available to study protein–protein interactions using biochemical approaches, the yeast two-hybrid system (Fields and Song 1989) has been the method of choice for genetic analysis of pairs of proteins that physically interact. The assay is simple and can be scaled to study a large number of interactions (for review, see Uetz and Hughes 2000). However, there are a number of reasons why similar genetic assays in *Escherichia coli* should be useful (Hu et al. 2000). First, *E. coli* has higher transformation efficiencies than *Saccharomyces cerevisiae*, allowing the construction of more complex libraries. Second, the endogenous proteins present in the cell can affect the assay. This is less likely to occur in a different cellular background like *E. coli*. Third, the yeast two-hybrid system requires nuclear localization of both prey and bait whereas a bacterial system would not, as bacteria lack nuclei. Until recently, bacterial two-hybrid systems that exploit these advantages were not available. During the last few years, however, several labs have described bacterial two-hybrid systems. Chapters 25, 26, and 30 describe two of the new bacterial systems. Others have been reviewed elsewhere (Hu et al. 2000).

This chapter describes an established system based on fusions to the amino-terminal DNA-binding domain of bacteriophage λ repressor, which has been used to examine a more specialized

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kind of protein–protein interaction: self-assembly into homotypic oligomers. Many proteins self-assemble into oligomers, and detecting and mapping oligomerization domains has become a standard part of structure–function analysis in the study of single-gene products. Genetic methods based on fusion proteins provide a convenient way to map and characterize oligomerization domains in the absence of an assay for the protein's normal function. Although some homotypic interactions are clearly detected by yeast two-hybrid systems, across the proteome yeast two-hybrid screens seem to underrepresent them (Hu 2000; Newman et al. 2000).

The activity of λ repressor depends on the presence of both the amino-terminal DNA-binding domain and the carboxy-terminal oligomerization domain (Pabo et al. 1979). Removing the carboxy-terminal domain reduces DNA-binding activity, inactivating the repressor (Fig. 1). However, repressor activity can be reconstituted when a heterologous oligomerization domain is fused to the amino-terminal domain (Hu et al. 1990). Since its introduction in 1990, the repressor fusion system has proven to be an easy-to-use genetic tool to study protein oligomerization in vivo. Bacteriophage λ repressor fusions have been used extensively to study the oligomerization properties of proteins from various organisms in *E. coli*. Repressor fusions have been used successfully to characterize oligomerization domains from bacterial proteins (Amster-Choder and Wright 1992; Turner et al. 1997; Kennedy and Traxler 1999; Jakimowicz et al. 2000), fungal proteins (Hu et al. 1990; Strauss et al. 1998; Nikolaev et al. 1999), plant proteins (Edgerton and Jones 1992; Gonzalez et al. 1997; Palena et al. 1997), insect proteins (Gigliani et al. 1996), and mammalian proteins (Lee et al. 1992; Romano et al. 1998; Tan et al. 1998). One feature of the repressor fusion system is its ability to distinguish between dimers and higher-order oligomers (Zeng and Hu 1997). This property of the system could be used to eliminate protein aggregates from the screen.

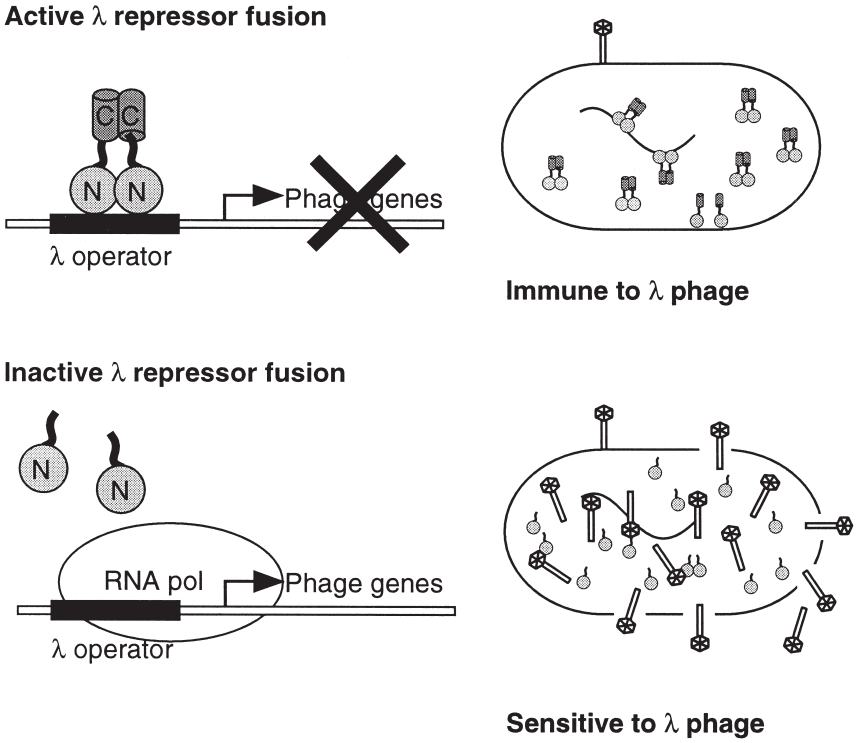

FIGURE 1. λ repressor fusions as a tool to study protein oligomerization. The λ repressor system is used to identify self-interacting domains. A positive interaction reconstitutes the activity of the repressor and the clone becomes immune to λ phage.

The use of repressor fusions has gone beyond the characterization of a single protein. Other applications include selections to disrupt interactions (Park and Raines 2000), design of peptide inhibitors (Jappelli and Brenner 1996), and characterization of proteins that require cofactors, posttranslational modifications, or molecular signals for oligomerization (Fiedler and Weiss 1995; Qin et al. 2000; Rashkova et al. 2000). Additionally, transmembrane segment interactions have been detected in vivo (Leeds and Beckwith 1998), opening new avenues for the study of interactions in nonsoluble proteins. Finally, library approaches include selections for high-affinity peptide ligands (Zhang et al. 2000) and selections to find novel self-assembly peptides encoded in *S. cerevisiae* and *E. coli* (Jappelli and Brenner 1999; Zhang et al. 1999).

Repressor fusions as well as other genetic methods to study protein–protein interactions have limitations; a genetic test can only suggest a physical interaction. Biochemical methods are then used to confirm the putative interaction. Like any genetic method, repressor fusions will miss certain interactions such as those that require posttranslational modifications for proper assembly. Therefore, repressor fusions should be thought of as providing complementary information to, rather than replacing, the ongoing large-scale two-hybrid screens (Fromont-Racine et al. 1997; Ito et al. 2000; Uetz et al. 2000) and other biochemical and genetic approaches (Phizicky and Fields 1995).

OUTLINE OF PROCEDURE

Whether the goal is to map a specific homotypic interaction from a known protein or to identify novel self-assembling domains, the repressor fusion system can be used in basically the same way. Because the characterization of self-assembling domains in known proteins has been the main application of repressor fusions, we focus below on how to find novel interacting domains from large-scale screens, such as a screen we have started in our laboratory for self-interaction domains from the yeast genome. However, the general approach for both small-scale and large-scale studies consists of the following steps (Fig. 2). First, individual clones or libraries are constructed from fragments of the target gene or genome fused to the amino-terminal DNA-binding domain of λ repressor. Second, selection and screening for repressor activity is carried out to identify fragments encoding self-assembling domains. Third, positive clones are identified by DNA sequencing and database searches to find the corresponding open reading frame (ORF) in the genome. Fourth, additional characterization can be done to examine the properties of any positive fusions.

Library Construction

Vectors

The repressor fusion vectors used for the identification of self-assembling domains are multicopy plasmids that allow cloning of the desired insert downstream and in frame with the DNA-binding domain of λ repressor. The identification of self-assembling domains requires low constitutive expression levels of the fusion proteins. This is because the amino-terminal DNA-binding domain when overexpressed is able to dimerize and thus confer immunity to phage infection even in the absence of its carboxy-terminal domain (Sauer et al. 1990).

There are three generations of repressor fusion vectors available in our lab. The differences are diagrammed in Figure 3. All of the ampicillin-resistant plasmids we use are descendants of pZ150 (Zagursky and Berman 1984), a pBR322 derivative with an M13 single-stranded (ss)DNA origin to facilitate M13-mediated transduction (Vershon et al. 1986). The region encoding the repressor fusion is placed between the *Eco*RI and *Eco*RV sites on the pBR322 map. The fusion protein is transcribed in the clockwise direction; a transcription terminator from the phage T7 ϕ10 gene is downstream of the cloning site. The plasmids also contain a deletion between *Bam*HI and *Sal*I sites in the *tet* gene that removes some restriction sites.

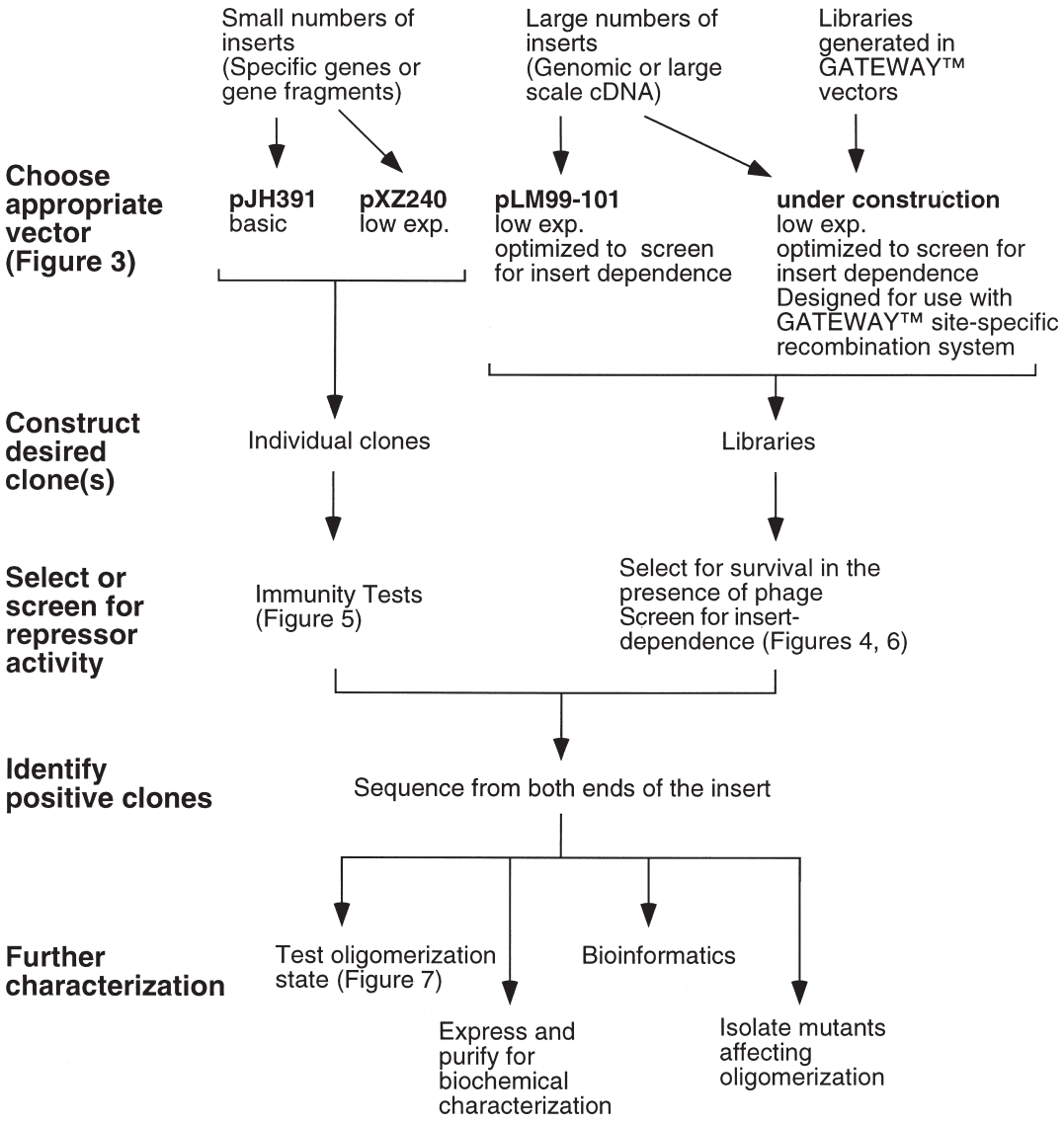

FIGURE 2. General scheme for characterization of self-assembling domains. Decisions about vectors to use and whether to use selections or screens are dictated by the source of the DNA to be tested. The figure shows different paths leading to the generation of active repressor fusions and their characterization.

In the original vector, pJH391 (Fig. 3B) (GenBank Acc. No. AF316554), the fusion protein was expressed from a minimal *lac*UV5 promoter. The 654 bp between the *Eco*RI site and the start of the repressor coding sequence contains the promoter region, a single *lac* operator, and the Shine–Dalgarno sequence from *lacZ*. There is no CAP-binding site in the promoter region. Constructs made using pJH391 must be used in a strain with the *lacI*^{q} allele, which overexpresses the *lac* repressor. The basal level of leaky expression is more than enough to give an immune (repressor-positive) phenotype.

In pJH391, the desired inserts can be cloned between a *Sal*I site and a *Bam*HI site. Because the plasmid was designed to accommodate a specific artificial gene cassette encoding the *GCN4* leucine zipper, polylinkers were not included. pJH391 contains a "stuffer" fragment between the *Sal*I and *Bam*HI sites that allows easier purification of backbone DNA cut by these enzymes. Note

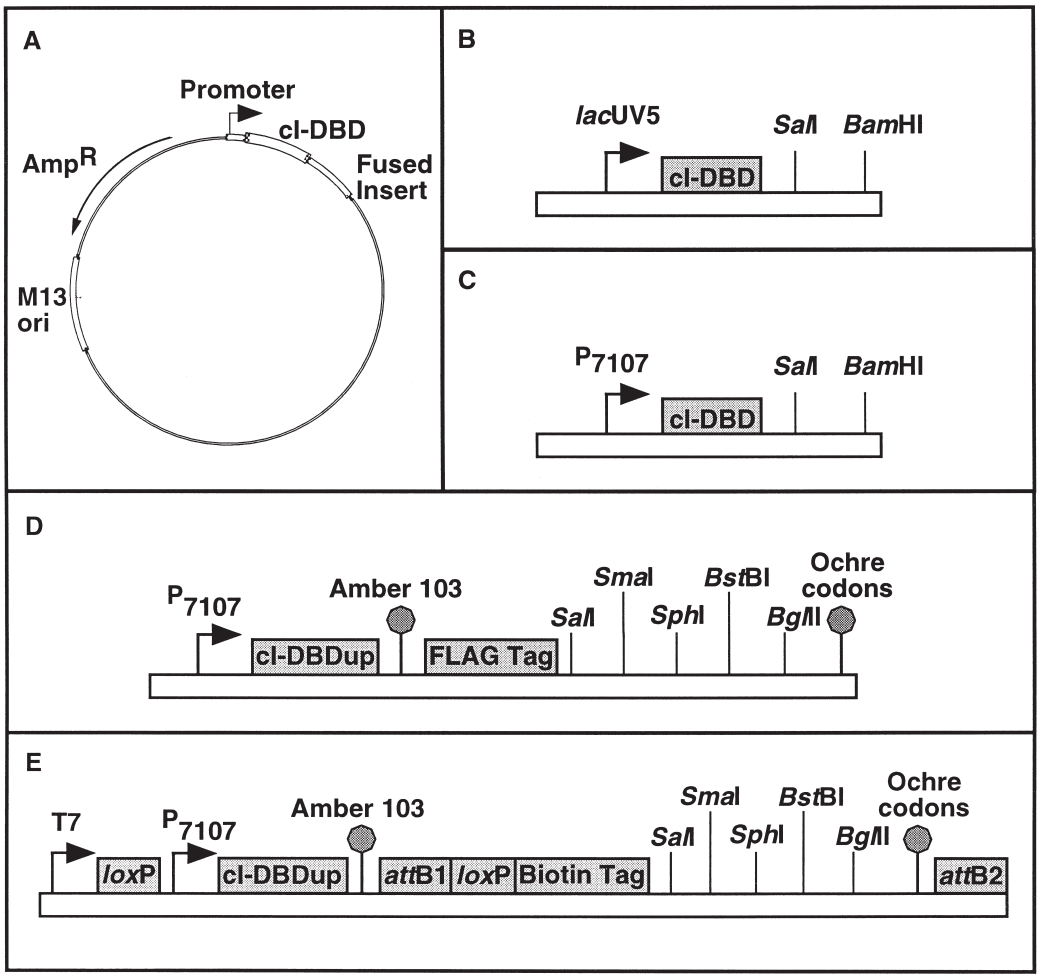

FIGURE 3. Important features in repressor fusion vectors. (*A*) Circular map indicating relevant features present in our repressor fusion vectors. Repressor fusions are constructed in a plasmid that contains an M13 origin of replication and an ampicillin-resistant marker. (*B*) First generation. A minimal *lac*UV5 promoter drives the expression of the fusions; inserts can be cloned using the *Sal*I and *Bam*HI sites. (*C*) Second generation. Repressor fusions are expressed at lower levels; P_{7107} replaces *lac*UV5. (*D*) Third generation. An amber mutation is introduced at position 103; additional unique restriction sites facilitate cloning. A translational terminator is introduced after the multiple cloning site (see text for details). (*E*) Fourth generation. Site-specific recombination will facilitate subcloning and overexpression of oligomerization domains. Segments are not drawn to scale.

that stop codons must be designed into the downstream end of the insert; readthrough into the vector can lead to the addition of a substantial amount of extra polypeptide, depending on the reading frame.

A kit based on pJH391 has been widely distributed and has been used successfully to study many different oligomerization domains. In 1997, we introduced a second generation of vectors (Fig. 3C). The main difference was the replacement of *lac*UV5 by a weak constitutive promoter called P_{7107} (Zeng et al. 1997). pXZ240 (GenBank Acc. No. AF316555) is identical to pJH391 except for the promoter replacement, which also introduces a unique *Xba*I site upstream of the cI start point. Operationally, P_{7107} seems to express the fusion protein at even lower levels than the basal level provided by *lac*UV5, even in the absence of isopropyl-β-D-galactopyranoside (IPTG); the actual differences in promoter strength have not been quantified. This lower level was impor-

tant to set up an in vivo assay for dimerization specificity based on negative dominance (Zeng et al. 1997). However, pJH391 is more permissive for weak oligomers and is still often the vector of choice for dissecting the oligomerization unit of a specific gene product.

To characterize oligomerization domains from a specific gene, one usually makes a series of constructs with different end points and then tests them by screening for repressor activity. When active fusions are isolated by selection, it is important to determine that positive candidates are displaying repressor activity due to self-assembly, rather than due to a mutation in the host or the vector (e.g., up-promoter or plasmid copy number mutations) that simply increase the expression level of the fusion protein. For our first- and second-generation vectors, this is done by recloning the inserts isolated by selection. However, recloning can become cumbersome for large numbers of clones. We are currently testing a third generation of vectors that allow a rapid assay for insert dependence (Figs. 3D and 4). These vectors contain an amber mutation at position 103 between the DNA-binding domain and the DNA insert. The resultant amber fragment, comprising residues 1–102 of λ repressor, has been shown to be stable in *E. coli* (Parsell et al. 1990). As illustrated in Figure 4, if the repressor-positive phenotype is dependent on self-assembly of the insert-encoded domain, it should be dependent on suppression of the amber mutation. This can be determined by M13-mediated transduction (Vershon et al. 1986) to suppressor (*supF*; LM58) and nonsuppressor (sup^0; LM59) strains (see below).

Our third-generation plasmids have multiple cloning sites to accommodate different kinds of DNA fragments. Our cloning site cassette contains recognition sites for *Sal*I, *Sma*I, *Sph*I, *Bst*BI, and *Bgl*II. In addition to DNA cut with the same enzymes, these sites can accommodate a wide variety of overhanging and blunt ends. Plasmids pLM99 (GenBank Acc. No. AF308739), pLM100 (GenBank Acc. No. AF308740), and pLM101 (GenBank Acc. No. AF308741) are third-generation repressor fusion plasmids with this cloning site cassette in different reading frames.

When using designed PCR fragments or synthetic cassettes to create the inserts, it is easy to control the reading frame and to make sure that there are stop codons at the end of the insert.

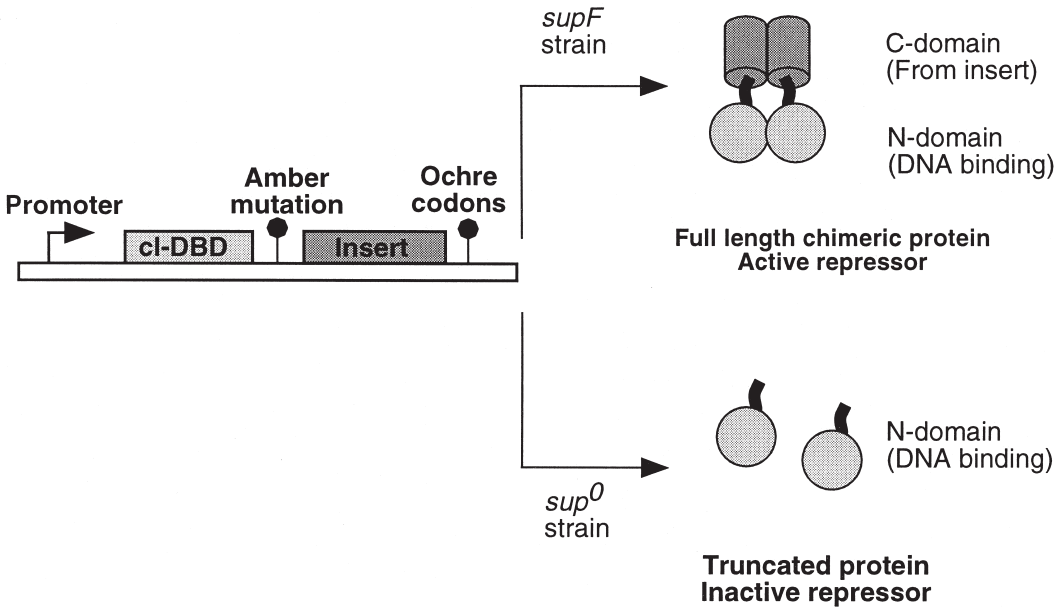

FIGURE 4. Nonsense suppression as a test for insert dependence. The presence of an amber mutation at position 103 allows a rapid screening for insert dependence using a reporter gene for the activity of the repressor fusion. In this particular case, an insert encoding a dimerization domain will display distinct phenotypes in suppressor (*supF*) and nonsuppressor (sup^0) strains.

When inserting random DNA fragments, it is important to prevent translation from vector sequences, which may contain fortuitous interacting sequences. The multiple cloning site is therefore followed by a series of stop codons in all three reading frames.

The third-generation plasmids also contain mutations in the DNA-binding domain that increase its ability to activate the P_{RM} promoter (Bushman et al. 1989) and a FLAG epitope tag in the linker between the repressor domain and the insert. The third-generation plasmids express the fusion proteins from the P_{7107} promoter. However, an additional deletion in the backbone increases the copy number of these plasmids; thus, expression levels are probably somewhat higher than in the second-generation plasmids.

Currently, we are developing fourth-generation vectors that will contain several other features to facilitate working with any self-assembling domains identified through large-scale library screening. In particular, we plan to build overexpression and purification features into the fusion vectors to allow biochemical characterization. We are also including site-specific recombination sequences to facilitate insert transfer to other kinds of vectors (Walhout et al. 2000). For example, this might be used to transfer an assembly domain identified in a repressor screen to an appropriate vector for expression in a eukaryotic cell, where it might generate a dominant negative phenotype (Herskowitz 1987).

The choice of which vector to use depends on the problem to be addressed. The higher expression level from *lac*UV5 can be useful for finding weaker oligomerization domains. For example, we have found that whereas the *GCN4* leucine zipper, which has a nanomolar dissociation constant, gives repressor activity when transcribed from both *lac*UV5 and P_{7107}, a construct containing the C/EBP leucine zipper only works when expressed from the *lac*UV5 promoter. However, the *lac*UV5 promoter is too active to use in the negative dominance assay, and using the *lac* promoter when selecting for active clones in a library can lead to false positives due to mutations in *lacI*.

Inserts

To find oligomerization domains in specific genes, restriction fragments or PCR products are generally used. For very small domains, such as leucine zippers, it is often practical to generate the insert entirely from synthetic DNA. When synthetic DNA is being used, convenient restriction sites can be designed into the insert to facilitate future mutagenesis studies. Several computer programs are available to identify places in a sequence where restriction sites can be introduced without changing the coding sequence. We have implemented a Web-based version of the program Seqsearcher (Reidhaar-Olson et al. 1991) at http://tofu.tamu.edu/seqsearch/.

For our studies on whole genomes, we have only used partially digested genomic DNA from organisms that have few or no introns. However, in principle, PCR products or cDNA can also be used as a source of inserts. For genome-wide studies, the construction of high-quality plasmid libraries is critical for the success of the screen. The domain responsible for oligomerization may be located anywhere within a protein, and many fusions may be nonfunctional due to frameshifts or insert orientation. Therefore, a highly representative library must not only represent each gene, but should also provide as many different fusion end points within each gene as possible. We fragment genomic DNA by partial digestion with the restriction enzyme *Cvi*TI*, which has the recognition sequence ~NGCN. This enzyme gives a quasi-random distribution of DNA fragments that can be used for shotgun sequencing (Fitzgerald et al. 1992; Gingrich et al. 1996). *Cvi*TI* generates blunt ends that can be cloned into the *Sma*I site of the repression fusion vectors.

E. coli *Strains*

In principle, a wide variety of *E. coli* strains can be used as hosts for the repressor fusion vectors. For the first-generation vectors, a strain with the $lacI^q$ allele must be used. If M13-mediated trans-

duction is going to be used, then strains must be F^+. In practice, we have found subtle differences in the behavior of control plasmids in different strains. For example, the introduction of reporter prophages seems to decrease repressor activity slightly; some constructs that are immune in an isogenic non-lysogen become sensitive in a lysogen. In contrast, some commercially available strains, including some XL-1 Blue derivatives, allow some of our negative controls, which lack a dimerization domain, to behave as if they are immune. This suggests that there may be differences in plasmid copy number or supercoiling among different laboratory strains. In addition, some strains contain unannotated mutations that affect sensitivity to λ or ϕ80. We recommend the use of AG1688 when the first and second generations of vectors are used. The third and fourth generations require an amber suppressor to obtain a full-length fusion; in this case we recommend the use of JH787 as the host for these repressor fusion vectors. Note, however, there are two disadvantages for these strains. First, neither is available commercially as competent cells. Second, AG1688 is *endA*$^+$, which means that plasmid preps from these strains should be deproteinized to prevent DNA loss due to endonuclease activity.

Selection and Screening

There are two classes of assays for repressor activity in *E. coli*: phage immunity tests and reporter constructs (Fig. 5). The ability of a strain expressing a repressor fusion to grow in the presence of phage can be used as either a selection or a screen. When testing a small number of specific constructs, screening is usually adequate. There are several simple assays for screening candidates. Cross-streak assays are done by placing a "line of death" on a plate by streaking a small amount of phage λ in a narrow line across a plate. Candidates are then streaked in single lines that cross the phage line at right angles. The plate is then incubated for anywhere from 5–6 hours to overnight. All candidates will grow up to the line of phage; only those restoring repressor oligomerization will grow through the line of death, whereas growth of sensitive cells will stop when they reach the phage. Individual clones can also be tested by examining the plating efficiency of phage λ. This can be done in several ways. The most rigorous way is to carefully titer a phage stock on the strain

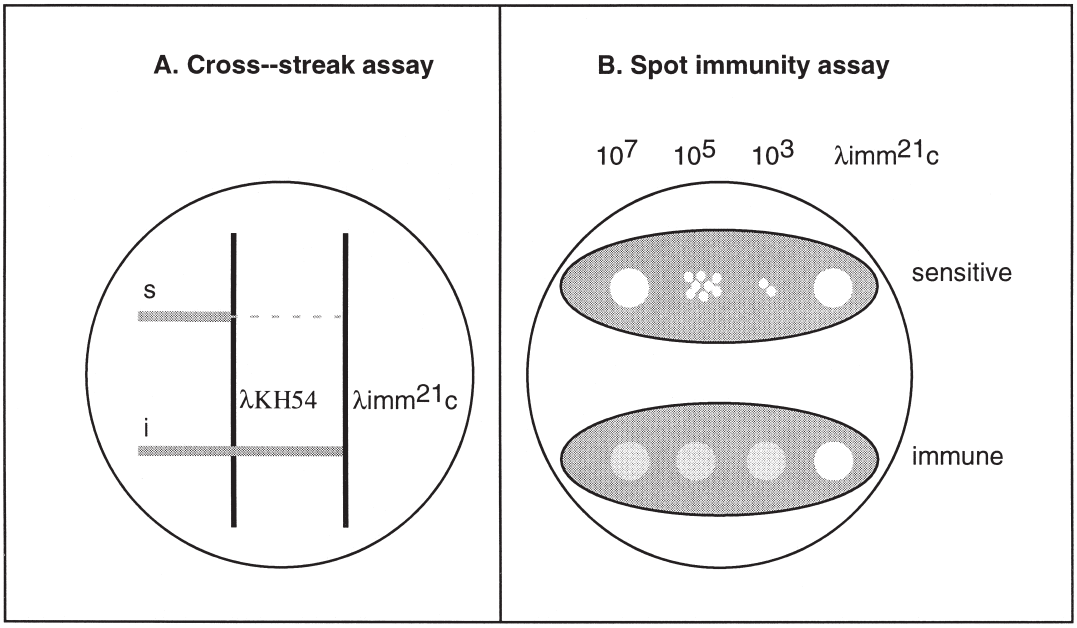

FIGURE 5. Simple immunity tests for repressor function. (*A*) Cross-streak assay. (*B*) Spot immunity test. See protocols for details.

of interest. An easier way to examine several candidates is to spot phage dilutions on preformed cell lawns in top agar. Selection for phage immunity is done by spreading cells on plates seeded with λKH54 and λKH54h80 phages. The KH54 mutation is a deletion in cI, which prevents the phage from forming lysogens. (A lysogen is a bacterial cell that has a prophage integrated in its chromosome; because λ lysogens express λ repressor and are immune to superinfecting phage, lysogens would show up as false positives.) The h80 phage uses the TonB protein as a surface receptor, whereas λ normally recognizes the LamB porin. A false positive could arise from mutations in recipient bacteria causing a loss of the surface receptor for phage attachment. Thus, by using a mixture of phages with two different receptor specificities, the background of survivors due to host mutations in receptor genes is reduced to a minimum.

Repressor activity can also be examined using a variety of reporter constructs that place a screenable or selectable marker under the control of λ operators. Operon (O) fusions that place the *lac* genes under the control of λ repressor were used to elucidate the regulation of the λP_R and P_{RM} promoters (Maurer et al. 1980; Meyer et al. 1980; Meyer and Ptashne 1980). For example, λ202 is a phage containing *lacZ* driven by $\lambda P_R O_R$. In this phage, O_R contains an $O_R 2^-$ mutation to eliminate cooperative binding by the wt repressor to the adjacent $O_R 1$ and $O_R 2$ operator sites. We have also constructed operon fusions where a *cat-lacZY* fusion is controlled by the λP_L promoter. In strains carrying this reporter, loss of repressor activity can be selected or screened using chloramphenicol (*cat*) resistance. Repression of this reporter is used in the screening strategy described below, to confirm the requirement for full-length fusions. Additionally, the β-galactosidase levels in the strains that contain these reporters can be determined by enzyme assays. However, whereas the difference between the repressed and unregulated levels is about 10-fold, the amount of enzyme made in the presence of an active repressor is still well above the level that gives a blue color to a colony on medium containing X-gal.

Selections based on negative control of a reporter, like the ones described above, are problematic. When a plasmid expressing a repressor fusion is introduced into a cell, the cell is already expressing the derepressed level of the reporter gene. If the reporter gene product is stable, its level is decreased only by dilution due to cell growth and division. This phenotypic lag means that libraries would have to be amplified before selection was applied. Selections based on activation of a reporter would not have this problem, because a small increase over the basal activity is easier to select, although it still takes several generations to reach steady state. In addition to its function as a repressor, λ repressor can act as an activator at the P_{RM} promoter (Meyer and Ptashne 1980). Although this activity should be useful, it has not been extensively exploited to examine repressor fusions. Note, however, that the phenotypic lag problem does not apply to phage selection because the phage promoters are not present before the repressor fusions are expressed.

Figure 6 shows a flowchart for the selection and screening of candidates from a genomic library using the third generation of vectors, illustrating the combination of phage selections and reporter constructs. The initial selection of candidates is for immunity to phage infection, as described above. Positive candidates are picked into wells in microtiter plates and grown in broth, which are used to generate M13 transducing stocks. The M13 stocks are used to do parallel transductions in microtiter plates into suppressor (*supF*) (LM58) and nonsuppressor (sup^0) (LM59) strains carrying the λP_L-*cat-lacZ* reporter phage. Transductants are patched onto plates with chloramphenicol and screened for drug sensitivity, which indicates that *cat* expression has been repressed. A desired candidate, where oligomerization is dependent on the insert, will be chloramphenicol-resistant in the nonsuppressing strain and chloramphenicol-sensitive in the suppressor strain.

Requiring a candidate to repress both phage infection and reporters is a stringent test. Although many repressor fusions are active in both kinds of screens, others act as repressors only in phage infection or only with reporters. The reasons for these differences are unclear. However, this may reflect the role of cooperative binding to multiple operators during phage infection, while the reporters have been chosen to reflect occupancy of single operator sites.

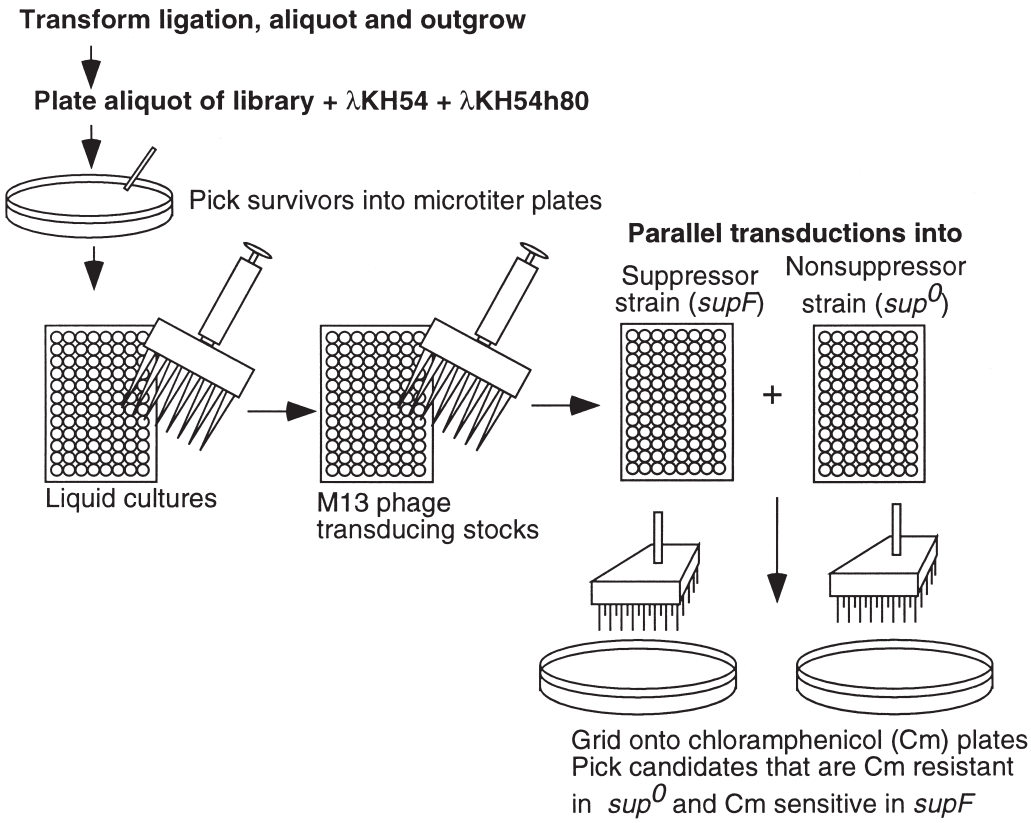

FIGURE 6. Flowchart for selecting self-assembling domains from genomic libraries. Clones are first selected on plates seeded with λ phage. Survivors are then transduced to *supF* and sup^0 strains, where clones expressing a repressor fusion encoding a putative self-assembling domain are identified. See protocol for details.

Self-assembling Domain Identification

Self-assembling domains isolated from libraries can be identified by DNA sequencing and database searches to assign the corresponding ORF in the genome. Plasmid DNA is extracted by a high-throughput method (Marra et al. 1999) and DNA sequence is obtained from both ends. We use two primers: the cI primer (5′-AGGGATGTTCTCACCTAAGCT-3′) reads clockwise starting in the linker region between the repressor DNA-binding domain and the insert and T-ϕ (5′-CTCAGCGGTGGCAGCAGCCAA-3′), which reads counterclockwise starting in the T7 gene 10 transcription terminator. Sequencing from both ends is important for the identification of chimeric inserts or peptides outside of the protein space. We use the BLAST (Altschul et al. 1997) programs to assign the positive clones to specific open reading frames (ORF), and the WebBLAST (Ferlanti et al. 1999) package to organize the reports generated by BLAST searches.

Testing Oligomerization States

Repressor fusions can also be used in a genetic assay to distinguish between dimers and higher-order oligomers (Fig. 7) (Zeng and Hu 1997). This assay is performed by comparing two strains. The first strain (JH607) contains two λ repressor operator sequences with different binding affinities: a proximal low-binding-affinity operator labeled "weak" and a distal high-binding-affinity operator labeled "strong." The second strain (XZ970) contains only the proximal low-binding-

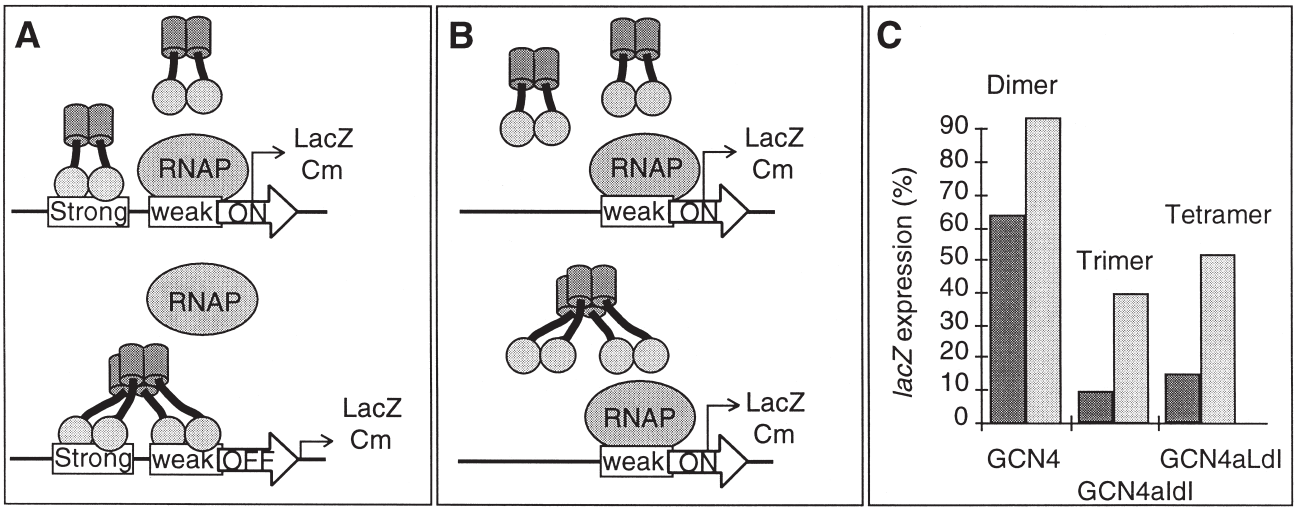

FIGURE 7. Genetic test to distinguish between dimers and higher-order oligomers. (*A*) Dimeric fusions will not fully repress reporter genes in the strain with two operators, whereas tetrameric fusions will bind to the two operators and fully repress the reporter genes. (*B*) In a control strain where only a single operator controls the reporter genes, both dimers and tetramers give comparable repression. (*C*) β-Galactosidase assays distinguish between dimers and higher-order oligomers. (*Light bars*) Single operator reporter; (*dark bars*) dual-operator reporter. (Adapted, with permission, from Zeng et al. 1997, © National Academy of Sciences, U.S.A.)

affinity operator. Reporter gene repression is achieved only when the proximal operator is occupied. Figure 7C illustrates how β-galactosidase assays can be used to quantify the difference in expression of *lacZ* for model leucine zipper dimers, trimers, and tetramers. Only trimers and tetramers are able to repress efficiently the *lacZ* reporter in the strain containing two operators due to cooperative DNA binding. This extension of the repressor system has also been used to examine other bacterial proteins besides a variety of λ repressor mutants and leucine zippers (Liu et al. 1998; Xia and Uhlin 1999), suggesting that other oligomers behave similarly in the system.

Protocol 1

λ Repressor Fusions

Preparation of individual clones or libraries in pJH391, pXZ240, or pLM99-101 should be done using standard molecular biology methods. Similarly, processing plasmid-containing strains for DNA sequencing can be done by a variety of standard methods (see Sambrook and Russell 2001, *Molecular Cloning*). Detailed protocols provided below are for methods that are either specific to repressor fusions or likely to be unfamiliar to labs not working with phage.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Solutions

Chloroform <!>

LB broth and agar (see Sambrook and Russell 2001, *Molecular Cloning*)

Premixed LB broth and agar are prepared according to the vendor's instructions.

Sodium citrate (1 M) (sterile)

TM buffer

10 mM Tris-HCl (pH 8.0)

10 mM $MgSO_4$ <!>

Autoclave.

Tryptone agar

Add 13 g of Bacto-agar per liter of tryptone broth before autoclaving.

Tryptone broth (per liter)

tryptone	10 g
NaCl	5 g

Add distilled H_2O to 1 liter. Dissolve, aliquot, and sterilize by autoclaving.

Tryptone top agar

0.7 g of Bacto-agar per 100 ml

We make this in bottles ahead of time and melt it in a microwave oven before use.

2x YT broth (per liter) (see Sambrook and Russell 2001, *Molecular Cloning*)

tryptone	16 g
yeast extract	10 g
NaCl	10 g

Dissolve in 1 liter of distilled H_2O. Divide into 100-ml aliquots. Autoclave.

E. coli Strains, Phage, and Plasmids

Strain	Genotype or description	Use	Reference
MC1061F$^-$	*araD139*, Δ(*ara-leu*)7697, Δ(*lac*)X74, *galE15, galK16, rpsL*(Str^R), *hsdR2, mcrA*, mcrB1	λ phage propagation	(Casadaban and Cohen 1980)
AG1688	MC1061 F'128 *lacI^q lacZ*::*Tn*5	host for first- and second-generation vectors; M13 transduction	(Hu et al. 1993)
JH787	AG1688 (ϕ80 Su-3)	host for third- and fourth-generation vectors; M13 transduction	(J.C. Hu, unpubl.)
LM58	JH787 (λLM58)	suppressor strain used for library screening of oligomerization domains	(L. Mariño-Ramírez, unpubl.)
LM59	AG1688 (λLM58)	nonsuppressor strain used for library screening of oligomerization domains	(L. Mariño-Ramírez, unpubl.)
JH607	AG1688 (λ112O$_s$P$_s$)	host used for testing oligomerization states	(Zeng and Hu 1997)
XZ970	AG1688 (λXZ970)	host used for testing oligomerization states	(Zeng and Hu 1997)

Phage	Genotype or description	Use	Reference
λKH54	λΔcI	λ phage used in selections and screens	(K.-C. Luk and W. Szybalski, unpubl. 1983)
λKH54h80	λ–ϕ80 hybrid, has the host range of ϕ80	λ phage used in selections and screens	
λimm^{21}c or λimm^{434}c	heteroimmune controls	used to test whether cells are sensitive to λ	
M13 rv-1	M13 helper phage	M13 transduction	(Zagursky and Berman 1984)
λLM58	λLM58 is λimm^{21} P$_L$-*cat-lac*; constructed by recombination between λXZ1 (Zeng and Hu 1997) and Plasmid pLM53 (GenBank Acc. No. AF179893)	contains the *cat* reporter used in library screens	(L. Mariño-Ramírez, unpubl.)

Plasmid	Description (see Fig. 3)	GenBank Acc. No.
pJH391	Amp^R, P$_{lac}$UV5-λcI[1–132]-*Sal*I-stuffer-*Bam*HI	AF316554
pXZ240	Amp^R, P$_{7107}$-λcI[1-132]-*Sal*I-stuffer-*Bam*HI	AF316555
pLM99	Amp^R, P$_{7107}$-λcI[1-116]-am103-FLAG-multiple cloning site cassette, frame 1	AF308739
pLM100	Amp^R, P$_{7107}$-λcI[1-116]-am103-FLAG-multiple cloning site cassette, frame 2	AF308740
pLM101	Amp^R, P$_{7107}$-λcI[1-116]-am103-FLAG-multiple cloning site cassette, frame 3	AF308741

Antibiotics

Ampicillin (200 mg/ml) in H_2O

1000× stock; use at a final concentration of 200 μg/ml.

Kanamycin (20 mg/ml) in H_2O

1000× stock; use at a final concentration of 20 μg/ml.

Chloramphenicol (25 mg/ml) <!> in 100% ethanol <!>
1000x stock; use at a final concentration of 25 μg/ml.
Gentamicin (15 mg/ml) (gentamicin sulfate) in ddH_2O
1000x stock; use at a final concentration of 15 μg/ml.

Special Equipment

Centrifuge (Sorvall Model RC-5B, Rotor SH-3000 or similar) for 96-well microplates
Heat block (Baxter Scientific Model H2025-1A)
Incubator (37°C)
Microplate replicator (96 pin; Boekel Model 140500)
Sterile toothpicks
Water bath (50°C)

METHODS

Preparation of λ Phage Stocks

λKH54 and λKH54h80 are used in both selections and screens for active repressor fusions. In this protocol, phages are plaque-purified before being amplified to generate working stocks.

On day 1:

1. Grow a 3–5-ml overnight culture of MC1061 in tryptone broth.
   Many other strains can be used to grow bacteriophage λ, but we prefer to use an F^- strain to grow our stocks to avoid the possibility of M13 contamination. Although fresh overnights are best, the same culture can be kept at room temperature and used for several days.

On day 2:

2. Melt and preincubate tryptone top agar at 50°C. It is important to let the agar cool to ~50°C before adding it to cells. If it is too hot, the cells will be killed. Prewarm three 100-mm-diameter tryptone agar plates to room temperature. Tryptone agar and top agar give better results than LB.
3. Make a set of serial tenfold dilutions of an existing phage stock (preferably an archival stock, not a repeated serial passage of a working stock) in TM buffer, such that the final dilutions have titers of ~100–500 pfu/ml. Prepare three 13 x 100-mm test tubes with 100 μl of MC1061 overnight and 100 μl of diluted phage from the last three dilutions. Incubate for 20 minutes at room temperature to allow the phage to adsorb to the cells.
4. Using a disposable 5-ml pipette, quickly add 3 ml of melted top agar to each of the tubes. It is not necessary to do anything to mix the agar with the cells and phage. Vortexing is counterproductive because it introduces bubbles into the top agar.
5. Immediately pour the top agar onto a plate and tip it back and forth to allow the top agar to spread over the surface. Do not overdo this; the top agar will solidify quickly.
6. Incubate overnight at 37°C.

On day 3:

7. Prewarm tryptone agar and tryptone top agar as before. Examine the plates from the incubator to find one with well-isolated plaques. Pick a single isolated plaque by stabbing it with a sterile Pasteur pipette. Move the pipette sideways to break the seal between the agar and the

bottom of the plate and withdraw the plaque as a plug of agar. Eject it into 0.5 ml of TM buffer. Add one or two drops of chloroform, vortex, and place on ice for a few minutes to allow the phage to diffuse out of the plug.

8. Transfer 0.2 ml of the "pickate" to each of two new test tubes. Be careful to avoid the chloroform. Vortex briefly to allow any residual chloroform to evaporate. Make a third tube with 0.2 ml of TM buffer as a control.
9. Add 0.1 ml of the MC1061 bacteria overnight to each tube. Preincubate, add top agar, and plate as before.
10. Incubate at 37°C until the plates with phage are covered with confluent plaques.

    Very small areas of the bacterial lawn should be seen where clusters of plaques converge. It may help to compare the phage plates to the control to follow the development of the uninfected lawn. This should take anywhere from 5 to 8 hours. Do not let the incubation go overnight; the titer will be decreased.
11. Add 5 ml of TM buffer to each confluent lysis plate. Leave overnight at 4°C in the dark to allow the phage to elute.

On day 4:

12. Transfer the liquid from the plates to a sterile screw-cap tube. A little less than 5 ml per plate will be recovered. Add a few drops of chloroform to kill any surviving cells.
13. Titer the phage stock using the same methods. Expect a titer of 10^9–10^{10} pfu/ml.

Screening by Cross-streak Assays

Cross-streak assays are a very fast and simple way to determine the immunity status of a particular fusion. This is usually the first test that is done once a fusion is constructed.

14. Using a marker, draw a pair of straight lines, about 1 cm apart, on the back of a LB ampicillin plate. Label one KH54 and the other imm21c (or imm434c, as appropriate).
15. Using a 0.1-ml pipette, a sterile stick, or an inoculating loop, paint about 5–10 μl of phage along the appropriate line.
16. Pick a fresh colony of a clone of interest with a sterile toothpick. Starting about 2 cm from the line of KH54, draw a single line of cells across both lines of phage.
17. Incubate for 6 hours to overnight at 37°C. Sensitive cells will die at the first line. Immune cells will cross the first line but not the second. Anything that crosses both lines has lost the λ receptor, or may be a non-*E. coli* contaminant.

    Results will vary depending on how heavy-handed you are when you pick the colony. It is better to examine the plates at earlier times, because at later times resistant cells can take over the streak. We recommend AG1688 or JH787 as the hosts for immunity assays.

Screening by Spot Immunity Tests

Plating efficiency can be measured by titering phage in parallel on a candidate strain and a strain with no repressor fusion. Spot titers are a rapid way to test large numbers of candidates; they can also be done in a way that uses fewer plates.

18. For each candidate, inoculate a fresh single colony into 2 ml of LB ampicillin. Grow for 8 hours at 37°C. For the best reproducibility, do not vary this outgrowth time.
19. Mix 50 μl of the culture with 2 ml of tryptone top agar. Use a disposable pipette to paint a sector of top agar onto a prewarmed LB ampicillin plate. We routinely use three or four different candidates per 100-mm plate.

20. Spot 2–3 µl of λKH54 diluted in TM onto the lawn. Use dilutions with 10^7, 10^5, and 10^3 pfu/ml. We use a multichannel pipettor to do the spots when there are a large number of candidates to process.
21. Incubate overnight at 37°C. Strains that do not show any plaques are considered immune. Strains with clear or turbid plaques are considered sensitive. We find that some strains give day-to-day variation in this assay.

M13-mediated Transduction

M13-mediated transduction is a simple, rapid, and inexpensive way to transfer plasmids containing the M13 ssDNA origin between F^+ cells. This can be used to test insert dependence (see below) or to transfer plasmids to reporter strains, such as those used to distinguish dimers from higher-order oligomers (Zeng and Hu 1997).

22. Use a single colony of cells containing each cI^+ fusion plasmid to inoculate overnight cultures of LB ampicillin. Prepare an overnight culture of the appropriate recipient strain by using a single colony of cells to inoculate overnight cultures in LB supplemented with appropriate antibiotics.
23. Mix 20 µl of the overnight culture with 2×10^{11} pfu phage M13 in 0.1 ml.
24. Incubate for 10 minutes at 37°C. Add 2 ml of 2× YT broth to the mixture and allow the cells to grow with aeration for another 8 hours or overnight.
25. Transfer part of the culture to a microfuge tube and spin down the cells for 10 minutes.
26. Transfer the supernatant containing the M13-transducing phage to a new tube and incubate for 15 minutes at 60°C to kill any remaining cells.

    The pasteurized phage stock can be stored for months to years at 4°C.
27. Mix 50 µl of the overnight culture of the recipient with 5 µl of each cI^+ fusion M13-transducing stock.
28. Incubate for 30 minutes at 37°C.
29. Spot 5 µl of each transduction onto a LB ampicillin plate. After the spot dries, use a toothpick to streak out from it for single colonies. The single colonies that grow should be struck for singles again the next day.

Selection and Screening of Candidates from Libraries Made with pLM99-101

Repressor fusions can be used to explore genomes for proteins that contain oligomerization domains (Jappelli and Brenner 1999; Zhang et al. 1999). The procedure below has significant improvements that allow the recovery of a wide variety of oligomerization domains with a low background (L. Mariño-Ramírez and J.C. Hu, unpubl.). Libraries are constructed using standard molecular biology methods (see Sambrook and Russell 2001, *Molecular Cloning*). The complexity needed for the isolation of oligomerization domains should be at least 10^6 independent clones. We recommend the use of JH787 as the host for selections.

1. For selection of immune clones: Plate ~10^8 JH787 cells containing fusion libraries on LB-ampicillin-kanamycin plates seeded with 10^8 pfu/plate λKH54 and λKH54h80. The libraries can be used for selection without amplification; this will increase the number of independent clones. However, the plating efficiency of immune clones will decrease because of the lag between transformation and achieving the steady-state level of a repressor fusion protein. Incubate overnight at 37°C.

2. Prepare three or more 96-well microplates (conical "V" bottom) for each library by adding 0.15 ml of 2× YT-ampicillin-kanamycin supplemented with 25 mM sodium citrate. The sodium citrate chelates divalent cations that would allow λ phage carried over from the selection plate to grow. Inoculate the wells with immune clones. Grow for 16 hours at 37°C.

   Keep using 96-well microplates for the rest of the protocol to facilitate manipulation.

3. Mix 5 µl of M13 rv-1 helper phage (~10^8 pfu) and 5 µl of overnight culture from immune clones. Incubate for 10 minutes at 37°C. Add 0.15 ml of 2× YT. Grow for 6 hours at 37°C.

4. Kill cells by heating for 20 minutes at 65°C. Centrifuge the plates at 1000*g* for 15 minutes. Store the plate, which contains 96 M13-transducing phage stocks, at 4°C.

5. For M13 transductions on LM58 and LM59 bacteria, mix 5 µl of M13-transducing phage and 50 µl of overnight culture from LM58 and LM59 (use different plates for each strain). Incubate for 30 minutes at 37°C. Use the microplate replicator to transfer the transductions to large 15 × 150-mm LB-ampicillin plates. Incubate overnight at 37°C.

6. From the LB-ampicillin plates, replicate onto LB-ampicillin-chloramphenicol plates using the 96-prong replicator. Incubate overnight at 37°C.

   A positive clone will be chloramphenicol-sensitive in LM58 but chloramphenicol-resistant in LM59. Expect about 50% clones that show a positive phenotype dependent on insert.

7. Prepare glycerol stocks from the clones transduced on LM58 by growing overnight cultures on LB-ampicillin and adding glycerol to 12%. Freeze at –70°C.

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